

Combating the exploiters of creationism

Creationism's resurgence and its exploitation by politicians pose challenges to scientists that cannot be ignored. More resolute activism is required if a decent scientific education is not to be denied to some young Americans.

Kansas state geologist Lee Allison gave American Geophysical Union (AGU) members a strong dose of reality last week about the resurgent creationist movement's success in eliminating evolution and aspects of the Earth sciences from the school curriculum in his home state, and how the political right in the United States is capitalizing on that Christian fundamentalist agenda (see page 847).

Scientists are easy targets, says Allison; they aren't organized to lead political battles, they aren't media savvy and they are easily pigeon-holed as members of a liberal élite. They are even easier targets for extremists than gays, he maintains, providing a telling political analysis of last summer's creationist victory in Kansas. The conservative wing of the Republican Party in Kansas that led the creationist drive originally planned to use gay rights as the central focus of its campaign, but that target was dropped in favour of scientists. After all, explains Allison, most cities in the United States have an annual 'Gay Pride' day or parade, but when was the last time people saw scientists proudly parading themselves as such in the streets?

Science Days, or even Science Pride Days, would not provide an adequate response to creationism or its political exploitation, but that scale of grass-roots organizing is what is required to bring rationality and intelligence to discussions over local school science curricula. New Mexico recently provided a blueprint of how scientists can succeed in the political trenches of America's neighbourhoods. Scientists organized, they walked precincts and, in October, got candidates elected to the state school board, which returned the teaching of scientific fundamentals to the state's school curriculum.

But such efforts — described as the new "cold war" by some scientists — are not necessarily easy. At a session on evolution at last week's AGU meeting in San Francisco, a scientist asked: "How do you fight creationists if you are a liberal atheist from New York?". That

question highlights the divide between some members of the scientific community and mainstream America today. Certain scientific discoveries, alarming to some of the public, are being manipulated by political forces — in particular, AGU panellists noted, the "Christian right" and conservative Republicans — so as to make science seem a threat to individual religious views in America.

Most scientists tend to avoid public confrontation. But the key to the battle for the teaching of good science in the presence of fundamentalism is to be more resolute in informing the public of the important role of science and actively to oppose the use of distorted perceptions of science as a political vehicle to create what one panelist last week called "the dark age" of Kansas. To that end, scientists need to reach out, to become involved in local school-board issues and to seek election to ensure appropriate scientific curricula.

Scientists new to the political trenches should not get locked into debates about creationist beliefs, which at the least can shift the focus to a form of intellectual sport and at worst collapse into fruitless opposed assertions or even pitched battles. Experience suggests that scientists should instead focus on the fundamental question: "Do people want good science taught in schools?", clearly and justifiably linking today's understanding of the Earth's history and of evolution inextricably with the science that will allow parents' offspring to be able to grow up and solve the problems of disease, to eat healthier foods that will prolong and improve their lives, and to reap the lifestyle advantages that technology brings.

As scientists rally in Kansas next year, hopefully to defeat the pro-creationist forces on the state school board, the plains state will be a political test for a new resolve among the US scientific community. But given the political forces riding the fundamentalist wave, it will take coordination, coherence and some powerful advocacy drawn from the ranks of many independently minded scientists to carry the day. ■

An ill-judged cut

A large reduction in German support for agricultural research in the developing world sets a bad example.

The turn of the year is a time for good resolutions, the arrival of the year 2000 even more so. Symbolically, therefore, Germany's decision to slash funds for the international system of agricultural research (see page 845) could not have come at a worse moment. What is much worse, it hits an agency that deserves every support. The Consultative Group for International Agricultural Research (CGIAR), established in 1971, has contributed substantially to many significant advances in agriculture that are likely to have saved the lives of millions.

But its work is far from finished. According to recent estimates, global food production will have to double within the next 30 years in order to meet the constantly rising demand. As agriculture in developing countries cannot be separated from environmental factors,

some of the organization's 16 institutes worldwide have in recent years carefully shifted their priorities towards research in the management of natural resources — a well-advised policy considering the rapidly growing number of people affected by water shortages. It should go without saying that these socially explosive challenges deserve major efforts in basic research and technology. The CGIAR's responsible development of partnership with the private sector further strengthens the case for its funding.

The international system of agricultural research will not collapse because of Germany's move — provided it is not imitated by others. Those targeting agricultural research in the developing world for cuts should bear in mind that such support is not only a question of morals and conscience. It is also an investment towards global peace. ■





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Concern as Germany cuts funds to agricultural research centres

Munich and Washington

Germany has sent shock waves through the international agricultural research community by making heavy cuts in its financial support for six high-profile agricultural research centres in developing countries.

The cuts were made last month following a decision to halve the DM35 million (US\$18 million) research portfolio of Germany's Ministry for Economic Cooperation (BMZ) over the next four years. They are part of a DM30 billion austerity programme introduced by the government to stabilize the country's economy.

Basic research carried out through the Consultative Group for International Agricultural Research (CGIAR), a network of 16 international research centres, will be particularly hard hit. In 1999, the BMZ contributed around \$17 million, of which \$6 million was for institutional core funding, towards the CGIAR's overall budget of \$345 million. Next year, the BMZ's contribution to the CGIAR's core funding will be reduced by \$2.5 million.

Six of the CGIAR's 16 institutes will carry the cuts. These are the International Maize and Wheat Improvement Center (CIMMYT) in Mexico City, the International Potato Institute in Peru, the International Center for Agricultural Research in the Dry Areas (ICARDA) in Syria, the International



Helping hand: Reeves (right) of CIMMYT, warns of serious risks to long-term research.

Crops Research Institute for the Semi-Arid Tropics in India, the West Africa Rice Development Association (WARDA) in the Ivory Coast, the International Food Policy Research Institute in Washington DC and the International Service for National Agricultural Research in the Netherlands.

CIMMYT, one of the world's leading grain-research organizations, will lose BMZ's contribution of \$400,000 to its annual core budget of about \$15 million. "This is a significant blow," says Timothy Reeves, who directs the centre, which has about 120 scientific

personnel in Mexico City and another 300 in 16 countries around the world. He says the cuts have meant that 14 scientific personnel are being made redundant at CIMMYT's headquarters at the end of the year.

Research at CIMMYT, founded in 1966, is widely credited with having helped to avert famines in Asia in the 1980s. Its scientists, in particular Norman Borlaug, the 1970 Nobel Peace Prize laureate, were largely responsible for the scientific breakthroughs behind the 'Green Revolution'. Today 40 per cent of CIMMYT's efforts are concentrated on Africa, providing underdeveloped regions with drought-tolerant maize.

Although CIMMYT will still be able to apply for project-based BMZ funds, Reeves is concerned that long-term scientific projects — such as research on wheat diseases — could be seriously at risk. He is also worried that Germany's decision could affect other nations. "Everybody is concerned," he says. "If Germany does this, what will other countries do?"

Some of the ambitious scientific projects pursued by the five other CGIAR institutes affected could also be damaged by the cuts. Marlene Diekmann, an expert in agricultural research at the Deutsche Gesellschaft für Technische Zusammenarbeit, a government-owned German agency for technical cooperation with developing countries, describes the cuts as "a disaster" for the whole system of international agricultural research.

Diekmann is particularly concerned that the cuts may damage some of the CGIAR's most valuable resources, such as ICARDA's gene bank, which holds more than 117,000 germplasm samples. ICARDA supplies more than 30,000 samples from this collection each year to the global research community.

"The maintenance of a gene bank is costly and cannot be financed by project-based funds alone," she says. She points out that successful long-term research projects strongly depend on reliable institutional funding. "Reductions in core funding are likely to result in a somewhat hasty chase for short-term success, whereas innovative and sustainable long-term research will probably be neglected," she says.

Dissection banned in Israeli schools

Jerusalem

The Israeli government last week announced a ban on animal experiments in the school system, specifically citing the dissection of frogs. Minister of Education Yossi Sarid last week said that there are now 'virtual' ways of teaching students anatomy, so that dissection is no longer justified.

But the ban and its potential implications immediately came under fire from Rami Rahamimoff, a member of the medical faculty of the Hebrew University of Jerusalem, and chairman of the ministry of education's advisory committee on biology teaching.

Rahamimoff said that the move will "dramatically affect the teaching of biology" and that frogs are not the main concern. He pointed out that dissecting frogs is not part of the compulsory curriculum, and that data from the ministry itself show that fewer than ten frogs a year are killed in schools.

His concern is that the sweeping language of Sarid's ban will include a more common kind of dissection, that of animal organs and fish, which biology teachers buy from butcher's shops. He is also worried that the ban will prohibit dissections by gifted high-school students enrolled in university biology courses.

Haim Watzman

The importance of the research carried out by the CGIAR is widely recognized. After last year's system review of the organization, a panel of international experts headed by Maurice Strong, the founding executive director of the United Nations Environment Programme, concluded that investment in the CGIAR had been "the most effective use of official development assistance".

Alexander von der Osten, executive secretary of the CGIAR, says that support from countries other than Germany has remained strong. "Our overall budget has been gradually going up," he says.

He adds that the CGIAR has appealed against the cuts to both the finance minister and the overseas development minister in Germany. "But we didn't obtain anything other than good words," he says. "We need to mobilize public opinion so that people understand that this research is in the interests of northern countries," he adds.

Ironically, the cuts coincide with a recommendation by the Friedrich Ebert Stiftung (FES), a foundation closely linked with Germany's ruling Social Democrat Party, to increase funding for the CGIAR. In a report on possible benefits of genetic engineering for agriculture in developing countries, published last week, the FES concludes that "cuts in public funding of agricultural research relevant to developing countries mainly hit the poor". **Quirin Schiermeier, Rex Dalton and Colin Macilwain**

NSF under fire in survey of customer satisfaction

Washington

Officials at the US National Science Foundation (NSF) are smarting at the results of a survey released last week that places it near the bottom of a list of government services ranked according to customer satisfaction.

Of 28 other participating programmes, only the Internal Revenue Service and the Occupational Safety & Health Administration came lower. Top of the list was the Administration for Children and Families.

The survey was sponsored by the President's Management Council and administered by the University of Michigan Business School. Replies to questions addressed to users of federal services were used to rank them on a 100-point index.

The survey asked grant applicants how well the NSF had handled their requests. Nathaniel Pitts, director of the foundation's office of integrative activities, says its 70 per cent rejection rate partly explains its low score of 57, compared to a mean of 68.6.

Of the 260 NSF survey respondents interviewed, 68 per cent had their grant applications rejected. But the answers to some questions in the survey have revealed

weaknesses that cannot be corrected merely by raising the acceptance rate.

Respondents gave the agency a low score for the timeliness and efficiency of the proposal process. They also gave low marks to questions on the quality of peer-review and the fairness of the decision. Pitts says he is most concerned about these measures, as peer review is "the cornerstone of NSF".

He adds that recent changes to the peer-review process — particularly new requirements that weigh the impact of proposed research on society — may have contributed to the low score.

Jack Crow, director of the National High Magnetic Field Laboratory in Tallahassee, Florida, which has received \$18.5 million in NSF funds this year, says he is a "happy customer" of the agency. But he agrees that NSF's peer-review system could be improved.

NSF officials are to carry out a survey to explore problem areas more fully, and promise to participate in the consumer satisfaction index survey again next year. The results of the survey are available at <http://www.bus.umich.edu/research/nqrc/govt.html>

Paul Smaglik

Japan to bring in mandatory tests for GM foods

Tokyo

Japan's Ministry of Health and Welfare (MHW) announced last week that tests on the potential health risks of genetically modified (GM) foods will be mandatory from April 2001. Such tests are currently carried out on a voluntary basis.

The ministry also announced that foods considered 'safe' would be labelled. But how this should be done is still under discussion, and the MHW says it is uncertain whether the labels would actually indicate safety, or whether they would take a similar approach to that announced earlier this year by the country's Ministry of Agriculture, Forestry and Fisheries (MAFF).

MAFF's draft regulations, released in August (see *Nature* 400, 605; 1999), require 30 food products containing GM ingredients to be labelled as such. Products with a mixture of GM and non-GM ingredients must be labelled as 'undifferentiated'.

Although the MHW hopes to include food safety in its labelling, many think that the two ministries should first unite their currently separate regulation systems.

The health ministry also intends to require more detailed tests on the possible



Keeping it 'natural': The Japanese public are growing hostile towards GM food.

toxicity and allergenicity of GM organisms. The current guidelines give only a general outline of the safety tests. In contrast, the proposed evaluation system gives specific details of the assessment procedures.

Mandatory testing of GM products means that the evaluation of new GM foods

would be halted until April 2001. This is expected to affect the commercialization of several products that have passed safety evaluation, including DuPont's soybeans enriched with oleic acid. But it may speed up the approval of GM products and improve their image with the public.

Most Japanese food manufacturers have abandoned GM ingredients. Major breweries, such as Kirin Beer, Suntory and Asahi Beer, have decided to make their beer GM-free. Ajinomoto, Japan's largest food-additive manufacturer, last week said it was to stop using GM soybeans in its products.

Kirin Beer has also abandoned its research into GM tomatoes, although the company says that it has spent very little money on the project, which uses technology developed by Calgene, the US agribiotechnology company.

Kirin no longer plans to do research on GM food, although it will continue research into other crops. Kagome, which makes processed food, says it has no plans to commercialize its products, but will continue its research. But Japan Tobacco hopes to bring to market its GM rice, which was approved in 1998.

Yoriko Uozum

Green light for neutrino beam to pass below the Alps

Geneva

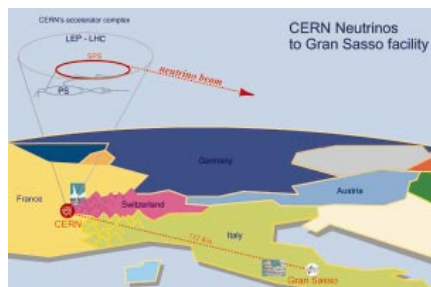
A major project to send a beam of muon neutrinos from Geneva under the Alps to the Gran Sasso laboratories near Rome was approved last week by the council of CERN, the Geneva-based European Laboratory for Particle Physics.

Italy's INFN, its national organization for particle physics, is organizing two experiments, known as OPERA and ICANOE, to study the oscillations of the neutrinos during their 730-km trip to Gran Sasso.

The experiments are designed to extend the breakthrough results achieved at the SuperKamiokande (Super-K) experiment in Japan last year, which showed that a proportion of muon neutrinos from cosmic rays disappeared, presumed to have been converted to tau neutrinos, as the beam travelled through the Earth (see *Nature* 394, 13; 1998). The Gran Sasso experiments will be optimized to detect tau neutrinos directly.

The neutrino beamline will cost SFr71 million (US\$45 million), of which INFN will pay around two-thirds. Special donations from other CERN member states, including Germany, Switzerland and France, will provide SFr16 million. First experimental results will be collected in 2005.

The idea has been under discussion for many years; Italian particle physicists have long been disappointed at being left behind. Japan and the United States are planning neutrino experiments using long-range



Direct route: the beam could solve some puzzles about neutrinos on its 2.5-millisecond journey.

accelerated beams in an attempt to repeat the Super-K results under more controlled conditions. But these will only look for disappearance of muon neutrinos, says Lorenzo Foa, professor of particle physics at the Scuola Normale Superiore, in Pisa, and a former director of research at CERN. "We will still need the positive confirmation of measuring the creation of tau neutrinos," he says, adding that Japan itself has expressed interest in participating in the experiments.

The approval also gives a shot in the arm to the INFN's laboratories in Gran Sasso. Built in the 1970s, they were deliberately orientated towards CERN in the hope that a neutrino beam would one day be created.

Luciano Maiani, the director-general of CERN, says that the quality of the planned neutrino beam and the innovative experiments being designed offer unique opportunities for science.

Alison Abbott

Scientists rally to defend schools against creationists

San Francisco

The American Geophysical Union (AGU) last week denounced the teaching of creationism and called for scientists to become politically involved in promoting the teaching of evolution.

The AGU council issued its 'advocacy statement' after a morning-long session at which scientists issued a call to arms to defend science teaching. More than 300 Earth scientists, including teachers, crowded into a session at the union's autumn meeting last week (see below), where they were urged to seek election to local school boards.

"The American Geophysical Union affirms the central importance of scientific theories of earth history and organic evolution in science education," reads the statement. "Creationism is not science and does not have a legitimate place in any science curriculum."

AGU president John Knauss said that creationists had become quite "clever" in distorting both science and the positions of scientific organizations. The union's previous statement was vulnerable in this regard, he added, because it was brief and did not sufficiently describe the AGU's opposition to creationism.

The AGU's new stance was prompted by the actions of two state school boards earlier this year. In Kansas, the board voted to cut the teaching of Earth science

Geophysics union entices biologists with new section

San Francisco

The American Geophysical Union (AGU) is setting up a new section for the biological sciences. The intention is to create a clearer focus and structure for incorporating biology into geophysical research and to attract biologists to the AGU.

Called 'Biogeochemistry, Biogeophysics and Planetary Ecosystems', the section is expected to advance research — particularly in global biogeochemical cycles and astrobiology — through scientific presentation posters, lecture meetings and publications.

It was approved by the AGU council during the union's annual autumn meeting last week in San Francisco. The meeting, attended by more than 7,500 scientists, included a biogeoscience programme that attracted more than 300 paper submissions. The council's required second vote is planned for the spring meeting in Washington DC.

A 13-member committee chaired by Diane McKnight of the University of Colorado had recommended creating the section. The panel said that improved coordination and articulation of the biological sciences "will foster a critical mass of researchers who will contribute the integrative links to advance our understanding of the Earth and planets".

The new section has been enthusiastically endorsed by AGU president John Knauss, a retired marine scientist associated with the University of Rhode Island and the Scripps Institution of Oceanography in San Diego, and president elect Marcia McNutt, a marine geophysicist who directs the Monterey Bay Aquarium Research Institute in California. "We must ensure that we are able to evolve as a society," said McNutt. "The science is changing. Biogeoscience is an example."

Not everyone was in favour. Committee member Dork Sahagian, an Earth scientist at

the University of New Hampshire, says that initially he was "dead set" against a new section, fearing it would "sequester" biological research. But he was soon persuaded it would integrate the disciplines. He then helped form, and presided over, a biogeoscience session on wetlands at last week's meeting.

Knauss says AGU officials have decided to "go back to the drawing boards" on a broader plan for reorganizing the existing ten sections. Robert Detrick, chairman of geology and geophysics at the Woods Hole Oceanographic Institution in Massachusetts, said the earlier plan was a complex formula to alter the scientific makeup of sections. He and others felt it was not sensitive enough to the needs of specific scientific disciplines.

"We need a mechanism to evolve over time," says Detrick, adding that more study is needed. A committee is being formed to work out an alternative plan.

Rex Dalton

and evolution from the state curriculum, which local school districts look to for guidance (see *Nature* 400, 701; 1999). In New Mexico, however, scientists and their supporters managed to get Earth science and evolution reinstated after creationists had had them removed from the curriculum.

Speakers at last week's meeting cited Kansas as an example of the scientific community failing to be sufficiently involved in fighting creationists, while the New Mexico campaign was held up as a demonstration of the role scientists can play in the political process.

By talking to voters, researchers from institutions such as the University of New Mexico convinced them to elect nuclear physicist Marshall Berman of Sandia National Laboratories to the state school board. This voted in October to reinstate Earth science and evolution. "We can work in the trenches to improve the quality of science education," said John Geissman, a geologist from the University of New Mexico. "It is being done; it can be done."

Meanwhile, an organization called Kansas Citizens for Science is campaigning to replace three pro-creationism school board members who are coming up for re-election.

One teaching assistant at last week's meeting said she was trying to find out how to deal with students who had been imbued with creationist philosophy.

Also at the meeting were two pro-creationist AGU members. One of them, John Baumgardner, a geophysicist at Los Alamos National Laboratory in New Mexico, asked for a dialogue within the union on creationism. He said that creationists had been represented as "simplistic characters". But he offered no rebuttal when geophysicist Wilfred Elders, of the University of California at Riverside, criticized a creationist college text on the purported rapid formation of the Grand Canyon that cited some of Baumgardner's work.

Rex Dalton



Spain's universities split over merits of performance table

Barcelona

A government-funded study that has ranked Spain's 44 public universities by giving each of them a score between one and ten has stirred up a fierce controversy.

Older universities came out best — seven of the top ten are at least 100 years old — and many of their officials say that the study confirms the quality of their work and shows the usefulness of comparative evaluation.

But younger universities, particularly those that are more technologically orientated, complain that the survey is based on outdated criteria. Young institutions accounted for eight of the ten universities that scored less than five.

The study was funded by the Ministry of Education and Culture. Scores were based on a survey by the National Statistics Institute. Six criteria were measured: educational development, organizational structure, teaching resources, participation of women, PhD activity and student success rate.

Carles Solà, rector of the top-scoring institution, the Autonomous University of Barcelona, says that his university's score of 8.5 "is congruent with previous data from diverse Spanish and European sources". He says that the evaluation process "may well open the doors to external observers in order to start objectives-based programmes".

Rafael Puyol, rector of the Complutense University of Madrid, which came second in the ranking, says that the result shows that, despite an "explosion" in student demand in recent years, "we have never overlooked quality". His university has more academic centres and students than any other in Spain.

The report has also been welcomed by Dario Villanueva, rector of the University of Santiago de Compostela, who says its results, "including our seventh position", are similar to those of an annual quality evaluation produced by a private company. The implication, he says, is that an evaluation culture already familiar in other countries "is beginning to emerge in Spain".

But Jaume Pagès, rector of the Polytechnic University of Catalonia in Barcelona, says the report is "inaccurate, unfinished, rash and lacking methodological rigour". According to Pagès, the quality of a university should be measured by whether "a series of internal and external objectives previously defined by the institution" have been reached — not through the use of indicators "arbitrarily established regardless of the university's goals".

He is particularly critical of the lack of external factors, such as those related to the social and economic environment or the



Seventh heaven? The University of Santiago de Compostela's rector is satisfied with its ranking.

number of students enrolled in international exchange programmes. Pagès says the report merely measures how close universities come to a "predefined, conventional, generalized and obsolete pattern", and adds that he is determined "to officially clarify problems involving quality in our universities".

Low-scoring universities have been especially critical of the failure to take account of links with private companies, for example through research contracts or patents.

Although the authors of the study say they were unable to include such a measure because of a lack of data, it appears to be a major reason why the 'technological' universities scored worse than expected.

The senior author of the study, Jesús de Miguel, director of the department of sociology and analysis of organizations at the University of Barcelona, and a social sciences consultant to the European Commission, has come under pressure from academics and the media. The rector of his university, Antoni Caparrós, has described the report as "not the work of my university".

Gemma Rauret, director of the public agency responsible for universities in Catalonia, points out that the report has not been submitted to experts' criticisms "but has sought media publicity". However, says Rauret, "its most positive aspect is that it has triggered a rethinking of the need to supply more and better information".

In response to the study, Saturnino de la Plaza, president of the Council of Rectors of the Spanish Universities and rector of the Polytechnic University of Madrid, says that the council is to set up a commission that will evaluate the quality of universities but will not provide any type of ranking.

De la Plaza says the study was biased against technological institutions. "You can only compare like with like."

Xavier Bosch

Top BSE official forced to step down in UK

London

One of the key government officials in Britain's recent crisis over bovine spongiform encephalopathy (BSE) has been forced out of his job. The move came days after the completion of an official inquiry that is expected to be highly critical of the way in which the crisis was handled.

The Ministry of Agriculture, Fisheries and Food (MAFF) announced on Monday the departure of Richard Packer, its top civil servant. Packer was at the centre of events in March 1996 surrounding the announcement of evidence that BSE might be responsible for a new form of Creutzfeldt–Jakob Disease (CJD) in humans (see *Nature* **380**, 273; 1996).

Ironically, Packer's departure coincides with new evidence — published this week in the *Proceedings of the National Academy of Sciences* by a team of US and UK researchers — strengthening the likelihood that new-variant CJD is caused by the same agent as that responsible for BSE in cattle.

This conclusion is based on the finding that mice injected with BSE-infected tissue develop almost identical symptoms to those of humans who develop the new-variant CJD, and over the same incubation period.

The study was conducted by researchers

in the University of California, San Francisco, Institute for Neurodegenerative Diseases, whose director, Stanley B. Prusiner, won the Nobel Prize in 1997 for discovering that spongiform encephalopathies were caused by prions. The new study used human brain tissue supplied by researchers at the National CJD Surveillance Unit in Edinburgh.

Packer — who describes his recreation in *Who's Who* as “living intensely” — is widely known as a highly skilful, respected and, in some quarters, feared civil servant. He has a reputation for staunchly defending the interests of his ministry (and Britain's agricultural community), and standing up to ministers when he felt this necessary.

Unlike most of his predecessors, he did not graduate from Oxford or Cambridge, but joined the ministry after postgraduate studies in chemical engineering at the University of Manchester in 1967.

His departure is being described by government officials as a reflection of their desire to ‘modernize’ the civil service, bringing it more closely in line with the strategic priorities of the Labour government.

But some say that Packer's position has been weakened by the evidence that has emerged over the past two years of the

BSE inquiry, headed by Lord Justice Phillips.

Questioning Packer on his earlier evidence during a cross-examination this month, for example, Phillips suggested that conclusions about a possible CJD/BSE link reached by the Spongiform Encephalitis Advisory Committee in February 1996 had not been treated with sufficient urgency.

However, Phillips also expressed concern that, when it becomes clear that the news had major implications both for public health and for the UK beef industry, “practical decisions were taken in a great rush”.

Packer has strongly defended his role in the crisis. He is reported in *The Times* to have told colleagues that he was only a “bit player”, and that “the inquiry will show that my actions have been close to exemplary”.

His departure after seven years in the top post at MAFF is likely to have major implications for the future of the ministry itself. Packer has long been a strong opponent of attempts to locate responsibility for food safety in a separate government department.

Such a move, he feared, could reduce what was until recently one of the most powerful ministries in Whitehall to little more than a Department of Rural Affairs. His departure makes that more likely. **David Dickson**

Middle East synchrotron crosses first funding hurdle

Paris

A scheme to build an international research centre in the Middle East using a synchrotron donated by Germany crossed a major hurdle last week. Eleven countries in the region agreed to provide sufficient funds to pay for dismantling the machine, now located in Berlin.

The German government had demanded that the project, overseen by the United Nations Educational, Scientific and Cultural Organization (Unesco), find the money to dismantle Bessy-1, a 0.8-GeV synchrotron radiation source, by the end of the year (see *Nature* **399**, 507–508; 1999). Bessy-1 needs to be moved to make room for the Max Planck Society's new history of science centre, and would otherwise have been scrapped.

During a meeting at Unesco headquarters in Paris last week, 11 countries each pledged US\$20,000 towards the project, known as Sesame (Synchrotron Radiation for Experimental Science and Applications in the Middle East). The United States will give another \$20,000, and Sweden will donate \$5,000. Unesco will provide the rest of the expected \$1 million needed to prepare the machine for shipping to a site yet to be decided in the Middle East.

Seven countries are vying to host the



machine — Armenia, Egypt, Iran, Jordan, Oman, the Palestinian Authority and Turkey. Israel is a member of the project, but is not competing to host the light source, as this would place the source off-limits to scientists from some neighbouring countries.

The decision about which country will host the machine will be based on criteria such as a central geographical location and openness to scientists of any religion, nationality or sex. Wealthier countries may have an advantage in the bidding, since the

new centre must have the financial means for its continued success.

Top candidates are said to include Jordan, the Palestinian Authority and Egypt. In contrast, Armenia, Iran and Oman may be too remote to provide easy access for countries in the area. And a few countries, such as Iran, have not received guarantees from their governments that the centre could guarantee broad access.

The next major hurdle will be to raise around \$100 million to build the new facility, to provide running costs for the first couple of years, and to upgrade the machine. An upgrade would include extending the machine's spectral range to 20–25 keV to provide hard X-rays, which are especially useful for protein crystallography and environmental studies.

Most of this sum will probably need to be raised outside the region, perhaps from large sources of funding such as the United States, Europe and perhaps even Japan.

“The money already coming to the region from other countries is very substantial, and we need only a few per cent of this,” says Herwig Schopper, former director-general of the European Laboratory for Particle Physics (CERN) and president of the project's interim council. **Heather McCabe**

South Africa doubles budget for medical research and AIDS

Cape Town The South African government is to substantially increase its investment in health-related research by doubling both the budget of its Medical Research Council (MRC) and its spending on AIDS vaccine development through the Department of Arts, Culture, Science and Technology.

The MRC budget will rise to 240 million rand (US\$39 million) over the next three years. The government will provide 65 per cent of this, with the rest coming from international private sources.

Research activities within the MRC are being reorganized into six main groups: ethics, HIV vaccine development, bioinformatics and human genomics, telemedicine, public-health research into the prevention of HIV/AIDS and tuberculosis, and research into crime, violence and injury.

The president of the MRC, William Makgoba, says the increase "is a sign that the government recognizes the vital link the MRC plays in national socio-economic development", and that the investment

"will improve the quality of life for all South Africans".

Safety at NIH labs to go under the microscope

Washington The US Health and Human Services Office of the Inspector General is to investigate biosafety standards at National Institutes of Health intramural laboratories. The move follows concern expressed by two members of the House of Representatives, Thomas J. Bliley (Republican, Virginia) and Fred Upton (Republican, Michigan).

The two had previously asked the office to move research on avian influenza from the Food and Drug Administration's labs in Rockville, Maryland, because the laboratory is near a shopping mall. The research is no longer being conducted at that location.

Ex-Lockheed boss to head Mars inquiry

Washington The US space agency NASA last week appointed Thomas Young to head the investigation into the Mars Polar Lander and Mars Climate Orbiter spacecraft, which were lost in recent months as they arrived at Mars (see *Nature* **401**, 415 & **402**, 565; 1999).

Young is a retired vice-president of the Lockheed Martin Corporation, which built and operated both spacecraft. He now chairs NASA's outside advisory committee on the international space station. The review panel will consider revisions to NASA's Mars exploration programme.

Earth Observing System takes to the sky at last

Washington The long-awaited Earth Observing System made its debut last week with the launch of NASA's Terra spacecraft into polar orbit. The first in a series of satellites designed to spend at least 15 years gathering data on the global environment, Terra will measure properties such as land cover and surface temperature, snow cover, ocean surface temperature, atmospheric temperature and humidity, and cloud and aerosol properties.

The instruments will be turned on during the first 11 days in orbit, although calibration and other start-up procedures will take about three months. The space shuttle also reached orbit on 19 December, carrying seven astronauts who will fix the ailing Hubble Space Telescope, out of service since last month because of faulty gyroscopes. The astronauts are due to release the repaired Hubble into orbit on Christmas Day.

Helicopter crash hits jinxed French lab

Paris The Institute of Millimetric Radio-astronomy (IRAM) has experienced its second fatal accident this year. Last week, a helicopter transporting five people on a service mission to the radiotelescope observatory crashed. The observatory, which is perched on the 2,550-metre-high Bure plateau in the French Alps, is operated by France's Centre National de la Recherche Scientifique, Germany's Max Planck Society and Spain's National Geographic Institute.

In June, a cable-car used to reach IRAM came loose and fell, killing all 20 people on board (see *Nature* **400**, 104; 1999). Since the accident, staff have used helicopters to reach the facility. The helicopter last Wednesday was carrying an IRAM technician, three employees of a subcontractor and the pilot. At least three of them are known to have died.

President's son promoted in Chinese academy

Beijing Jiang Mianheng, son of Chinese President Jiang Zemin and former head of the Shanghai Institute of Metallurgy of the Chinese Academy of Sciences (CAS), has been appointed a vice-president at

the academy. Jiang received his PhD from Drexel University, Philadelphia, in 1991.

Jiang has a reputation for successfully transferring technology to industry, an ability much needed by the CAS, which is traditionally dominated by basic research. His companies at the metallurgy institute earn millions of dollars each year in income.

Meanwhile, Xu Zhihong, a prominent physiologist and a CAS vice-president, has been named president of Beijing University.

Seeburg rapped over falsified data

Munich German scientist Peter Seeburg has been formally reprimanded by Hubert Markl, president of the Max Planck Society (MPS), for misrepresenting data in a paper published in *Nature* in 1979 (see *Nature* **399**, 512; 1999). Seeburg, a director of the Max Planck Institute for Medical Research in Heidelberg, had admitted the falsification.

In a lawsuit over patent rights on growth-hormone DNA from the University of California, San Francisco, Seeburg testified that he had taken DNA from the university in 1978 to the US biotechnology company Genentech. The case was settled last month (see *Nature* **402**, 335; 1999).

According to a committee set up by the

MPS last spring, a lack of regulation at UCSF in 1978 made it unclear whether taking the samples was illegal or not. But given his testimony about the 1978 paper — which is challenged by his co-authors — the committee concluded that Seeburg is guilty of scientific misconduct.

The committee said a reprimand was “sufficient and appropriate” as the case dates back 20 years. Seeburg will remain a Max Planck director — and he will also receive US\$17 million as co-owner of UCSF's growth hormone patent.

Extra money for Biosphere 2 provides new lease of life

San Diego Columbia University trustees have approved a \$50 million plan to expand teaching and research facilities at the Biosphere 2 centre outside Tucson, Arizona (see *Nature* **402**, 567; 1999). The domed enclosures at the ‘Columbia West’ campus are used as laboratories for Earth-science research and for teaching up to 350 students.

The plan includes a 10-year agreement between Columbia, in New York, and Texas billionaire Edward Bass. Columbia intends to pump about \$20 million more into the facilities, officials say, while Bass is pledging \$30 million.

Patent law changes spell problems and opportunities

Sir— Software can now be patented in the United States, and earlier this year a court decision lifted the ban on patenting 'methods of doing business'. These moves could have an important impact on scientists — many programs and protocols routinely used in laboratories may now qualify for patent protection.

Over the past 20 years, the US courts have gradually lifted the constraints against patenting software. The argument against patentability was that software is an arrangement of algorithms, similar to any mathematical formula or abstract idea, that cannot be removed from the public domain. But there existed a recognition that software could embody novel and non-obvious inventions which should be the protectable fruit of an inventor's labour.

To try to clear up the confusion, legal tests were established that allowed one to claim a patent for the application of a mathematical algorithm or abstract idea if it brought 'a useful, concrete and tangible result'. There was general feeling that the application of an algorithm had to be in the context of a machine or similar transformation of physical quantities. One could claim a machine, or method of operating a machine, that applied a mathematical algorithm, formula, or calculation with the practical result of generating an electrical signal or image on a display. It was also acceptable to claim software when reduced to a fixed medium, such as a computer disk. But these tests were cumbersome and confusing, especially where the application of a program produced a pseudo-abstract result such as a set of numbers.

Any question as to the patentability of software was eliminated by the decision in *State Street Bank v. Signature Financial Group, Inc.*¹. That decision was re-emphasized in *AT&T Corporation v. Excel Communications, Inc.*².

At issue in the *State Street* case was the patentability of a system for pooling mutual fund assets in an investment portfolio. The court confirmed that the system was patentable and held that the "transformation of data by a machine through calculations into a final share price, constitutes a practical application of a mathematical algorithm, formula, or calculation, because it produces 'a useful, concrete and tangible result' — a final share price momentarily fixed for recording". One can surmise from this that the requisite useful result of the application of an algorithm by a machine running software need only be a number in electronic form.

The courts did not stop there. There had been a general understanding that methods of doing business were not patentable. It appears now that these are as patentable as other processes or methods, subject to the requirements of novelty and non-obviousness. Arguably, such methods could include a host of laboratory protocols and data manipulation routines run on computers.

In the *Excel* case, even the requirement that a machine be implicated in a data processing operation was essentially eliminated. The issue was the patentability of a process using Boolean logic to create data structures for telecommunications switching applications. The court found that the bare process of using Boolean principles to produce useful, concrete and tangible results, without pre-empting other uses of these mathematical principles, was patentable. The need to claim a process involving a mathematical principle within the constraints of the operation of a machine was discarded.

To the scientific researcher, the *State Street* and *Excel* decisions present a two-edged sword. On the one hand, novel and non-obvious software test protocols, data reduction programs, calculation schemes, and numerous other computer-based manipulations of formulas, which once were not thought patentable, arguably now qualify for patent protection. On the other hand, one must now be wary of infringement of the patent rights of others in inventions that may apply well-known algorithms or calculations.

Businesses are becoming aware of the possible impact of these decisions. A deluge of patent applications, for such diverse business methods as Internet advertising and invoicing systems, is arriving at the US Patent and Trademark Office. I fear that the patent office will be ill-equipped to examine such applications, especially for inventions in fields where algorithms are widely used and accepted as conventional, and therefore not regularly described in print.

James J. Murphy

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1. 149 F.3d 1368, 47 USPQ2d 1596 (Fed. Cir., 1998).

2. 172 F.3d 1353, 50 USPQ2d 1447 (Fed. Cir., 1999).

Latecomers welcome?

Sir— Your supplement "Career choices for scientists" describes some of the interesting moves out of the lab that researchers make from necessity or choice (*Nature* 402 (Suppl.), 11 November 1999). Does anyone do the reverse? Are there scientists who acquired a science degree after their mid-twenties, say, but who made it into full-time research? With the

huge growth in education for mature students in the past 30 years there are many thousands with degrees acquired later rather than earlier. Do significant numbers of them reach the research lab?

David Davies

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Threat to research in Spanish universities

Sir— To help solve their chronic funding deficit, Spanish public universities are encouraging staff to bring in resources by collaborating with the private sector. This was made possible in 1983 by article 11 of the University Reform Law, which permits faculty members to sign contracts with private institutions. As an incentive, such contracts may include personal remuneration. The aim of article 11 is to promote collaboration between universities and industry through scientific and technical research projects. But illegitimate application of the law is eroding this philosophy, to the detriment of the scientific objectives of universities.

Article 11 is increasingly being used to conceal contracts offering ordinary services of low technical complexity, that have nothing to do with research and fall within the scope of private companies. These activities largely use infrastructure and staff paid for by public funds. In this way, the university is stealing employment opportunities and competing with the private sector. So universities may be undermining one of their main functions: the integration of qualified young people into the labour market.

The provision of these services by universities is also opposed to their primary objective as institutions dedicated to basic science. Such contracts, often given priority because they help line the pockets of the staff, take up precious time that would be better spent on research of wider scope and scientific value. The incorrect application of article 11 is leading to an illicit trade in which unscrupulous companies transfer routine activities to university labs, thereby reducing production costs, and academic staff obtain easy money without having to compete for funds with other researchers on the basis of proper scientific evaluation.

This will slow down scientific progress.

It would be intolerable if universities became no more than vulgar enterprises supplying everyday technical services. Article 11 contracts must be regulated to ensure that they promote scientific excellence.

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Seeing both the woods and the trees

A novel approach to classifying biodiversity may aid attempts at conservation.

Terrestrial Ecoregions of North America: A Conservation Assessment

by Taylor H. Ricketts *et al.*

Island Press: 1999. 485 pp. \$75, £50 (pbk)

Stuart L. Pimm

Metaphorically, population ecologists study single species of trees, community ecologists study the woods that are the collections of tree species, and ecosystem ecologists view woods and trees as so many kilograms of carbon. While important questions fall neatly within these divisions, the continuing destruction of biodiversity imposes questions across their boundaries. 'Biodiversity' means the full variety of life — between individuals of a species, the different species themselves, and the different assemblages they create. In practice we count and map species. Documenting within-species variability is too daunting a task for

all but a few species. And no two ecologists agree on what constitutes an assemblage. It can be an 'association', 'habitat', 'ecosystem', 'biome', 'life-zone', 'landscape' or 'biotype'. Neither do they agree on where, say, the woodland biome ends and the grassland biome begins. Unfortunately, the hegemony enjoyed by species creates its own problems. Perhaps only 15 per cent of all species have names, and we have range maps for only a tiny fraction of them. How can we conserve biodiversity when its measures are either arbitrary or incomplete?

The solution most often found in the pages of this journal is to test whether small samples of known species are representative. They are not. Peaks of species richness of one species group rarely match the richness peaks of another group. Neither do they match the 'hotspots' — the concentrations of endemics, those species with the smallest geographical ranges and therefore greatest

vulnerability. Ricketts and his colleagues have chosen the less trodden path, bravely classifying the United States, Canada and Mexico into about 150 inevitably arbitrary "ecoregions". In doing so, they follow their parent organization, the Worldwide Fund for Nature (WWF), in expressing concerns about purely species-based exercises in setting priorities. Importantly, this book documents the patterns of species within ecoregions and so uniquely integrates the two views of biodiversity.

A comparison of WWF's list of 200 global priorities and the list of hotspots drawn up by Conservation International or BirdLife International uncovers many identical choices. It is the differences that are interesting. Florida's Everglades or Brazil's Pantanal, for example, do not rank as hotspots, but achieve prominence because flooded grasslands are globally scarce and uniformly vulnerable. Other regions attain prominence because of the biological phenomena they house, such as the Arctic tundras and their migratory shorebirds and caribou. Most of this book describes the individual regions, the features that make them biologically distinctive, where the largest blocks remain, and what threatens them. No eco-traveller should leave for an ecoregion without it.

However one may debate the lines drawn between ecoregions, they indisputably provide a more compelling base onto which to map the patterns of species richness and endemism than political boundaries or lines of latitude and longitude. On a larger geographical scale and with more taxa than previous studies, the book documents these patterns. Butterflies, tiger beetles, reptiles, birds and mammals have similar patterns of richness, concentrating in the southwest. Base your conservation priorities on these groups alone, however, and you miss the Appalachian concentrations of amphibians, snails and vascular plants, the southeastern concentrations of trees and the western concentration of conifers.

Idiosyncratic patterns of richness and endemism provide a strong argument for an ecoregional approach: "We cannot wait until we have mapped out many taxa, nor for taxonomists to complete the species catalogue. (The end of this new millennium at the current rate of progress.) We know an ecologically distinct region when we see one and we see its threats. Seeing the woods is enough; don't sweat the trees." Indeed, the summary list of a dozen key regions provides compelling priorities not found in other exercises. The more pluralistic and more inclusive ecoregional approach may better anticipate



The renaissance of the plant

The tradition of illustrated herbals, or herb books, established in ancient Greece by Diokles of Karystos around 350 BC, was revived during the German Renaissance by the "German fathers of botany", most notably Otto Brunfels and Leonhart Fuchs.

The use of printed woodcuts greatly helped the dissemination of knowledge of plants in medicine during the fifteenth century, although these early illustrations were mostly useless for the purposes of identification. But it was the invention of the printing press that paved the

way for the golden age of the herbal. Fuchs' *De historia stirpium commentarii insignes* (Notable Commentaries on the History of Plants) was published in 1542, a year before and equally illustrative of the Renaissance as Andreas Vesalius' *De humani corporis fabrica libri septem* (see overleaf). A historical and botanical commentary by Frederick G. Meyer, Emily Emmart Trueblood and John L. Heller accompanies the facsimile edition of Fuchs' masterpiece (*The Great Herbal of Leonhart Fuchs*, Stanford University Press, \$249.50, £185).

hitherto unknown patterns of species richness and endemism than those we know from the few well-studied taxa. Moreover, it is readily exportable to continents where even fewer species are known and mapped.

Ecologists readily erect ad-hoc explanations for these patterns. Unfortunately, none of the explanations predicts which taxa should be similar and which different. I may believe the explanation of why snails have an inordinate fondness for Appalachia, but what in that explanation tells me that it is one that amphibians share, but not reptiles? Surely we must integrate what we know about how trees create a wood with the history of how the trees got there in the first place. Reading this book is a great way to start such integration. Its conservation message is a powerful enjoiner to do so immediately. ■

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Italian art's bequest to science

La Ragione e il Metodo. Immagini della Scienza Nell'Arte Italiana dal XVI al XIX secolo [Reason and Method. Images of Science in Italian Art From the 16th to the 19th Centuries]

edited by Marco Bona Castellotti, Enrico Gamba and Fernando Mazzocca
Electa: 1999. 206 pp. DM118

Pietro Corsi

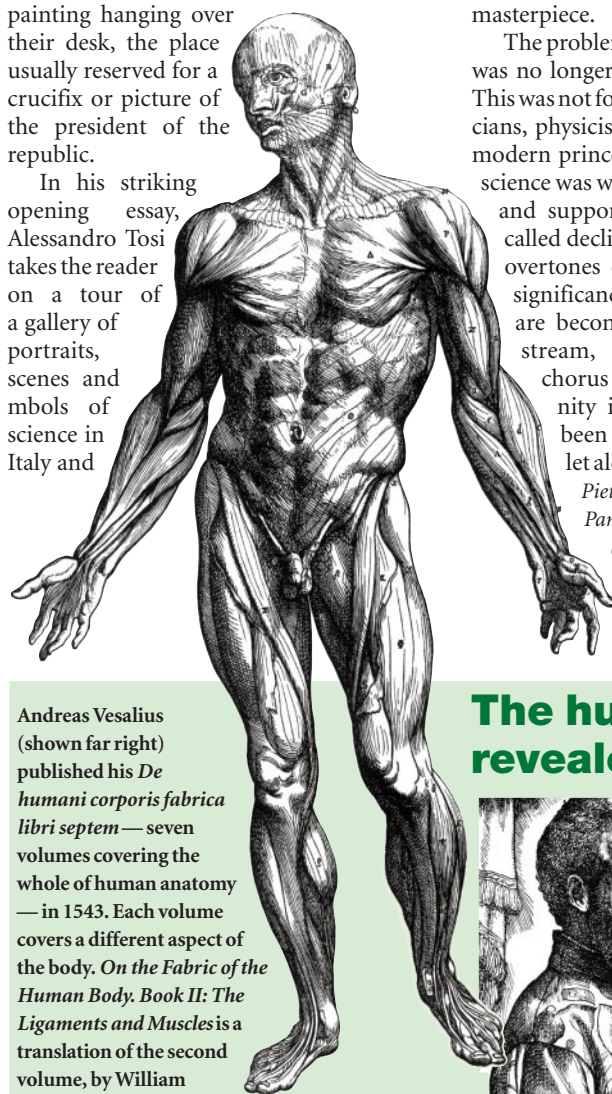
For the past 20 years or so, historians of science have paid renewed attention to the role of images in the development of modern science and medicine. True, the splendid tables of Andreas Vesalius' *De humani corporis fabrica* have always elicited admiration and stimulated research. But the recent revival in interest has seen many exhibitions and books, such as the 1994 exhibition *L'âme au corps*, shown at the Grand Palais in Paris, co-authored by Jean Clair and Jean-Pierre Changeux, and devoted to the relationship between science and the arts from the 1790s to today.

La Ragione e il Metodo is the permanent legacy of another exhibition, smaller in scope, but no less ambitious. It started with the decision by the city council of Crema, a wealthy town in northern Italy, to celebrate the 90th anniversary of the death of Giovanni Vailati, a mathematician and philosopher of science who was active at the end of the last century and the beginning of the present one. This was a further instance of the cultural ferment still to be found in provincial Italian towns willing to undertake

ambitious scholarly programmes.

As the title of the exhibition's catalogue suggests, the focus of the exhibition was the "images of science in Italian art, from the 16th to the 19th centuries". In other words, the ways in which scientific developments were represented in paintings, and in their full ideological, polemical and allegorical robes. Since Galileo's trial for heresy and his subsequent condemnation, Italian science has been at the centre of a long-lasting, at times patchy, ideological war. Indeed, one of the most famous paintings on show, the *Portrait of Galileo* by Giusto Suttermans, completed in 1635, until recently conveyed a message that transcended both science and art: you could tell a lot about a person's intellectual and ideological position if you knew whether or not they had a copy of the painting hanging over their desk, the place usually reserved for a crucifix or picture of the president of the republic.

In his striking opening essay, Alessandro Tosi takes the reader on a tour of a gallery of portraits, scenes and mblols of science in Italy and



Andreas Vesalius (shown far right) published his *De humani corporis fabrica libri septem*—seven volumes covering the whole of human anatomy—in 1543. Each volume covers a different aspect of the body. On the *Fabric of the Human Body*. Book II: *The Ligaments and Muscles* is a translation of the second volume, by William Frank Richardson with John Burd Carman (Norman Publishing, \$250). It highlights the skills of Vesalius the dissector, and contains all the well-known full-length muscle plates (above). These take the reader through a dissection sequence that unveils the human body layer by layer. Vesalius' detailed instructions for their use are given in full.

Europe. The scope of the catalogue, although focusing on Italian painters, is not limited to science on the peninsula. Thus, Giovanni Battista Pittoni at the end of the eighteenth century, and Pelagio Pelagi in 1827, paid homage to Isaac Newton through powerful pictorial inventions. However, from the second half of the eighteenth century and throughout the nineteenth century, Italian artists generally looked less kindly on the sciences. It was their French, British and American colleagues—from Joseph Wright of Derby and Jacques-Louis David to Henry William Pickersgill and Charles Wilson Peale—who took it upon themselves to continue the tradition that had begun in Italy with the extraordinary *Portrait of Luca Pacioli* (attributed to Jacopo de' Barbari, not without Tosi's scepticism) and Vesalius' masterpiece.

The problem was, and still is, that science was no longer a key cultural issue in Italy. This was not for lack of first-rate mathematicians, physicists and biologists. Rather, the modern prince—the state—did not feel science was worthy of consistent patronage and support. And today, with the so-called decline of ideologies, even the last overtones of the Galileo affair and its significance for the country's history are becoming submerged in a mainstream, vociferous, post-modern chorus deprecating scientific modernity in a country that has never been allowed to become modern, let alone 'scientific'. ■

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The human body revealed



Reflections on colour constancy

Karl R. Gegenfurtner

A study of colour perception shows that, when assigning colour to objects, the seeing brain takes into account subtle reflections of light between the surfaces in a scene.

For more than two centuries, scientists and artists have come up with a range of ways to demonstrate that the wavelength composition over a whole scene can affect how we perceive the colour of the individual parts of that scene. The proportion of light of each wavelength reflected from an object can be highly beneficial in detecting or recognizing objects¹. However, to make use of that invariant, the visual system somehow has to discount the illuminating light, which can vary quite drastically.

This process is usually called 'colour constancy', and the degree to which it is shown depends on many factors. Some of them occur at the early stages of sensory processing², such as local colour contrast, whereas others (for instance, colour memory) occur at higher cognitive levels³. Most computational schemes for achieving colour constancy try to decompose the overall light reaching the eye into one component that is due to the illuminant, and a second component due to the reflectance.

However, the physics of light is more complicated than the simple reflection of light from a surface into the eye, as if looking at a photograph. In a three-dimensional world, some light is reflected from one surface, but it then bounces to yet another surface from which it is reflected into the eye (Fig. 1). And so on. These indirect reflections are called 'inter-reflections', and are of especial interest to those involved in computer graphics and computer vision⁴. For example, computer simulations of indoor scenes appear more realistic when inter-reflections are taken into account. Indeed, many recent advances in computer graphics are due to the discovery of efficient algorithms to calculate the effects of all such multiple bounces of light among the vast number of surfaces typically contained in a scene.

Reporting on page 877 of this issue, Bloj *et al.*⁵ tested whether inter-reflections are taken into account by the human visual system when perceiving the colour of surfaces. To do this, they presented subjects with a folded card, with its two sides opening towards the observer (Fig. 2). One side of the card was painted magenta, the other white. The magenta half reflects light only in part of the spectrum, some of which reaches the eye directly, and some of which is reflected onto the white side of the card. As a result, the light reflected from the white side is no longer

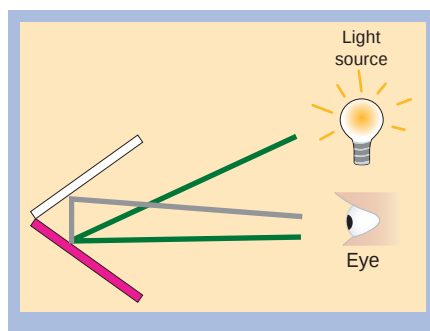


Figure 1 Illustration of inter-reflections. Part of the light is being scattered directly into the eye of the observer (green line). Other light rays enter the eye after being reflected from the magenta side of the card onto the white side (grey line). The magenta is 'bleeding' into the white.

balanced over all wavelengths, and it should appear a quite saturated magenta under the conditions of the experiment. Surprisingly, subjects reported that it appeared only slightly pinkish, suggesting that the visual system discounts the effect of inter-reflections to compute surface reflectance. In other words, the colour we perceive is influenced not only by the two-dimensional image of an object projected onto the retina, but also by our perception of the object's three-dimensional shape.

The strength of Bloj and colleagues' paper lies in the way they controlled for potential confounding factors. They used a special device called a pseudoscope, which optically inverts the depths of all objects in the scene. This means that the card will appear to open away from the observer when seen through the pseudoscope (Fig. 2b). This leads to an identical two-dimensional distribution of image intensities over the visual field. Perceptually, however, there is no longer the possibility for inter-reflections between the two surfaces, because they no longer seem to be pointing towards one another.

In this way, the physical stimulus was unchanged, so the inter-reflections and their effect were still present. But they could no longer be interpreted as such by the brain. Instead, the bias in the wavelength distribution of the card seemed to be due to its surface reflectance, and, as a result, it was perceived to have a deep pinkish hue. So the results show not only that three-dimensional shape can affect colour perception, but also

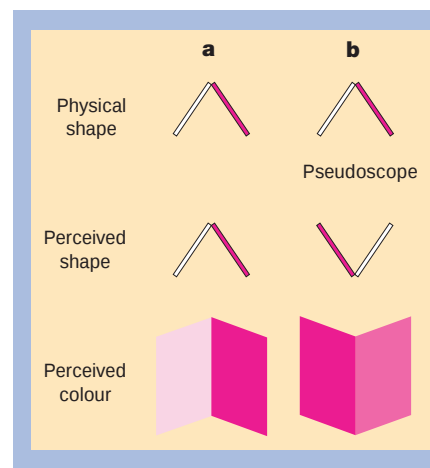


Figure 2 The experiment done by Bloj *et al.*⁵. Observers viewed a folded card opening towards them. a, Under normal viewing, observers discount the effect of inter-reflections. b, When seen through a pseudoscope, the inter-reflections are interpreted as a change in surface colour because they are incompatible with the percept of the bent-away card. The magnitude of the effect has been emphasized for clarity.

that the seeing brain indeed knows the physics of light inter-reflections.

Similar phenomena are well known in our perception of brightness. When dealing with black and white scenes, the effects of shape, reflectance and illumination are basically interchangeable, presenting the visual system with an infinite number of possibilities for interpreting such scenes⁶. Yet we perceive the world as being quite stable. The solution seems to be that the most likely percept is calculated, so changes in the perception of three-dimensional shape can easily (and profoundly) affect our perception of brightness⁷.

The question is, then, how much knowledge of the physics of light has the visual system acquired? It seems to know even the very subtle aspects, which may have only a very limited function when viewing natural outdoor scenes. For example, the geometric information contained in highlights — generated by mirror-like reflections from glossy surfaces — can affect the perception of surface curvature⁸. In their experimental set-up, Bloj *et al.*⁵ chose conditions that emphasized the role of inter-reflections. The white side of the card almost lay in the shadow, so it received very little direct light; most light

came from the magenta card. In typical real-life situations, however, a much smaller part of the light reaching the eye is due to inter-reflections.

A tremendous amount of psychophysical characterization has been done on visual 'illusions', such as those mentioned above, yet we know hardly anything about the brain processes that underlie such phenomena. The analysis of colour and other elementary features such as motion or orientation is often assumed to occur at an early stage of sensory processing in the visual system, whereas information about surfaces and objects is thought to be extracted during the later stages. Bloj and colleagues' results are difficult to reconcile with this simplified idea. One has to differentiate between the different uses of colour information in visual perception. Colour might be used at an early stage to segment objects from one another and from the background, but at that stage there is no need to assign a definitive colour to each object. Higher visual areas (such as the infero-temporal cortex, which is known

to be important for object recognition⁹) also show a large degree of colour selectivity¹⁰, and Bloj and colleagues' results support the idea that the colours we perceive may be determined at a rather late stage of visual processing.

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Photography

Making every photon count

Richard Hailstone

More than 150 years after the invention of photography, the underlying solid-state photochemistry is still being investigated and improved. One aspect of continual importance is the efficiency of the image-recording stage, which strongly affects the sensitivity of the photographic emulsion. Photons falling on a silver halide microcrystal in the emulsion cause a halide ion (such as chloride or bromide) to lose an electron, which is then captured by a silver ion, converting it into a neutral silver atom. The build-up of silver atoms gives rise to silver clusters: the 'latent image', which can be developed after several more steps into a print¹. In reality, the halide ions generate pairs of electrons and holes (the positively charged counterparts of electrons), and it is generally accepted that recombination of photogenerated electrons and holes is a major loss process^{2,3}. On page 865 of this issue, Belloni *et al.*⁴ present an ingenious way of overcoming this recombination process by doping the silver halide with formate ions, which leads to an increase in the number of silver atoms generated per photon absorbed.

Image-forming reactions in silver halide microcrystals compete with various electron-loss processes, the dominant one being recombination. Overall, many of the photogenerated electrons are lost and the sensitivity of the silver halide emulsion is low. The sensitivity can be greatly improved by chem-

ical sensitization, which is how all modern films and print materials are prepared. During chemical sensitization, reagents containing labile sulphur and gold atoms are used at low concentrations (parts per million or lower) to produce silver-gold sulphide clusters on the surface of the microcrystal. These clusters appear to deepen the existing traps for electrons, thereby reducing recombination^{5–7}.

During film development the silver halide microcrystal is converted to metallic silver through an electrochemical reduction reaction catalysed by the latent image. A microcrystal can only be developed when the latent image contains three or more photogenerated silver atoms⁸. In the theoretical limit, one photon should produce one silver atom. So the most sensitive film imaginable will only require three photons per microcrystal to create a latent image. Despite the advantages of sulphur-plus-gold sensitization there is still some recombination, and three to ten times as many photons are needed^{9,10}. So reaching this theoretical limit requires an additional mechanism to eliminate the remaining recombination. In the past this has been done by reduction sensitization, which chemically creates silver clusters on the surface of the microcrystal. These silver clusters have different properties from the photogenerated silver clusters in that they trap and destroy holes through an

oxidation process at the silver cluster¹¹. In model systems (but not in commercial films) reduction sensitization, in addition to the sulphur-plus-gold sensitization, allows one to reach the theoretical limit^{9,10}.

Unfortunately a significant level of fog is often a byproduct of the combined sensitization processes. Fog is the result of latent-image formation in the absence of light exposure and, when present, reduces the dynamic range of the material for image capture. In addition, the stability of chemical reduction is often low, so it is not usually a viable option for photographic manufacturers. Placing the chemically produced silver clusters inside the microcrystal could eliminate the fog problem and presumably would stabilize the sensitization. But this may change the electronic properties of the silver clusters, because some experiments indicate that they have electron-trapping properties that would actually decrease the sensitivity¹².

Efficiency improvements are important to photographic manufacturers because efficiency is a factor that determines the film speed. Light absorption also determines film speed and this aspect is controlled by the size of the microcrystal. This is the usual way in which different film speeds are obtained.

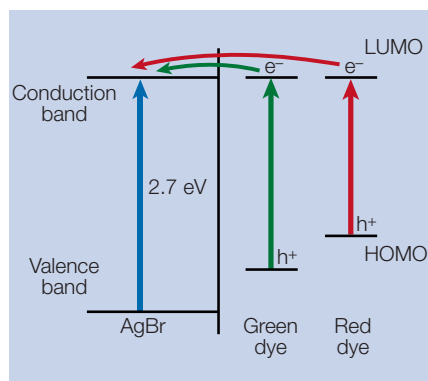


Figure 1 Colour photography requires three layers of silver halide emulsion sensitive to blue, red and green wavelengths of light. The effect of adding a green- or red-absorbing dye to AgBr (which absorbs blue light) is shown here. Absorption involves movement of an electron from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO), leaving a hole in the HOMO level. The photoinduced electron transfer occurs between the LUMO and the AgBr conduction band, leaving behind an oxidized-dye radical. The LUMO levels for both the green-absorbing dye and the red-absorbing dye are positioned at the conduction-band minimum so that efficient electron transfer can occur. Thermal energy can be used to move the hole into the valence band, thereby preventing recombination with an electron. Belloni *et al.*⁴ show that formate doping increases the sensitivity of green-dyed AgBr. Improving the overall efficiency of colour films might be possible, if similar increases in sensitivity can be shown for red-dyed emulsions.

Unfortunately, the noise in the final image also increases with microcrystal size¹³. So, higher and higher film speeds can be reached, but the image tends towards lower quality, particularly when enlargements are made. Increasing the efficiency of latent-image formation is a much better, although more difficult, way to achieve higher film speed.

The work of Belloni *et al.*⁴ shows one way to do this without incurring problems related to reduction sensitization. The use of formate (HCO_2^-) as a dopant is a novel way to overcome the residual recombination in conventionally sensitized microcrystals. Not only does it appear to be efficient at destroying holes, but the resulting unstable CO_2^- radical is able to provide an extra electron for latent-image formation. Now the theoretical limit becomes two silver atoms per photon. Similar results have been observed with reduction sensitization, but never without some risk of fogging^{10,11,14,15}.

Silver bromide emulsions, such as the one studied by Belloni *et al.*, only absorb blue light. This is because quantum theory dictates that discrete (rather than continuous) energy levels are formed in the crystal lattice, creating a permitted 'conduction band' for free electrons with the right energy. To capture and display the full colour of the original scene it is necessary to use spectral sensitization in addition to chemical sensitization. In spectral sensitization, dyes are adsorbed on the microcrystal surface and provide absorption in the region of the spectrum where the microcrystal is unable to respond¹⁶. The absorption event places an electron in a higher energy state, from where it can be transferred to the conduction band of the microcrystal and then participate in the usual process of latent-image formation (Fig. 1). Unfortunately the dye also introduces an energy level for trapping holes, making it more difficult for the formate ions to destroy them.

If formate doping is to be effective in increasing the sensitivity of dyed microcrystals it is necessary for the photogenerated hole to transfer to the valence band of the silver bromide crystal. Otherwise holes trapped by the dye act as recombination centres. Belloni *et al.* show that the hole will move into the valence band for a dye absorbing in the mid-green region, although the sensitivity increase is less than what they see with undyed microcrystals. One might suspect that this lower degree of efficiency is due to hole trapping by the dye so that holes are less likely to be destroyed by the formate dopant. Furthermore, one might expect the degree of sensitivity improvement to be even less for dyes absorbing in the red region, where holes are even more deeply trapped (Fig. 1). Studies with red dyes will be important for assessing the general value of formate doping as a way to remove residual

inefficiencies in the photographic process and achieve the highest sensitivity possible. ■

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Neural networks

Evolutionary checkers

Igor Aleksander

It was headline news in May of 1997 when the IBM computer 'Big Blue' beat chess champion Gary Kasparov. So why is it that Kumar Chellapilla of the University of California in San Diego and David Fogel of Natural Selection Inc. have written two papers^{1,2} in which they suggest that this seeming triumph "is testament to the limitation" of the very idea of artificial intelligence?

Artificial intelligence is often hailed as a new development, but the year 2000 will see its fiftieth birthday. In 1950 Bell Laboratories engineer Claude Shannon wrote the first chess-playing program for a computer. This

allowed computers to hatch out of the shell of numerical calculations and perform tasks that could be said to require logical 'thought'. Shannon showed³ that the game of chess could be reduced to numerical manipulation through the invention of a 'minimax' algorithm that allowed a computer to choose a good move without having to evaluate all the states in the intervening moves. This became proof that the number-crunching abilities of computers could be used to perform tasks which, if done by humans, would be said to require intelligence.

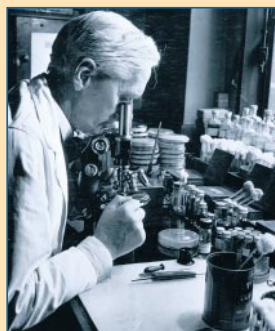
Chellapilla and Fogel's critique is based

Ergonomics

Suffering for one's science

Was Alexander Fleming a martyr to backache? If this picture is anything to go by, he probably was — along with 80% of long-term microscope users. They are also afflicted with eye strain, a variety of muscular pains and tension headache. Help is at hand, though, from Alfons Kreczy and his colleagues (*Lancet* **354**, 1701–1702; 1999).

Kreczy *et al.* designed a workstation to straighten out the hunched posture of microscopists. They adapted the eyepieces so that they could be used with only a 5° downward tilt of the head. They built an elevated table for the microscope with supports for the forearms. And they installed a chair that encouraged an



upright sitting position and supported the lower back.

They then compared the muscle activity of users of the new, improved workstation with that of people sitting in an ordinary chair and using an ordinary microscope perched on a pile of books. The ergonomic workstation resulted

in a big decrease in the activity of the most strained muscle groups — those of the neck, arms and lower back. Unfortunately, eye strain is an unavoidable consequence of scanning lots of slides.

Maintaining an unhealthy posture over a long period results in pain starting more quickly and taking longer to go away. Eventually it will carry over into rest periods and even, research on postal workers has found, into retirement.

So if you find yourself getting up from your microscope, clapping one hand to your forehead, the other to your back, and emitting a heartfelt groan, you might want to show this copy of *The Lancet* to your boss. **John Whitfield**

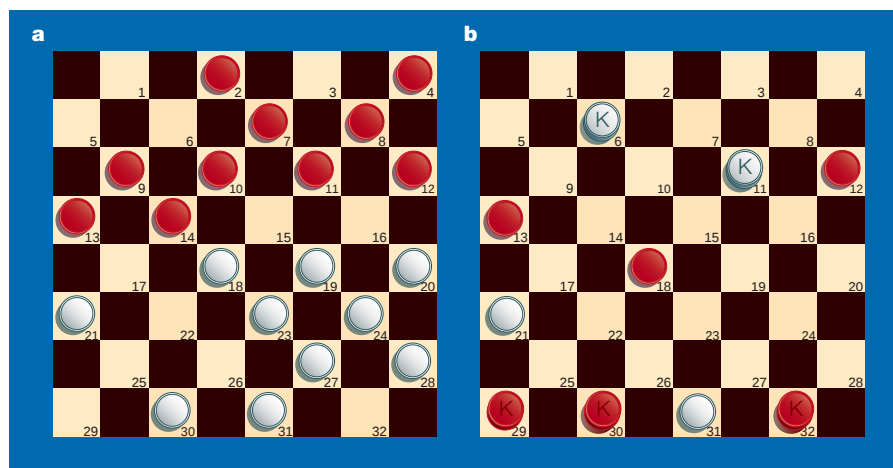


Figure 1 A checker-playing neural network from generation 230 of Chellapilla and Fogel's evolutionary series takes on a human player, and wins. a, A turning point in the game comes at move number 11 when white (the human) walks into a trap set by red (the neural network). Red moves 11→16; white is then forced to move 20→11, whereupon red strikes: 8→15→22. White is one down and never recovers. b, The endgame. It is move 32 and white is now down by a piece and a king (K). Red has just moved 14→18, and white's pieces on 21 and 31 are pinned down. With an endgame of three kings (red) versus two kings (white) in sight, white resigns. (Redrawn from ref. 1.)

on an approach that avoids the need for a programmer to work out the game-playing program. It uses evolution in artificial neural networks which, they argue, is an advance both for artificial intelligence and the design of such networks. The proposed artificial evolution encourages the survival of those systems in a batch that exhibit success in beating artificial opponents. New batches are derived from successful networks and the process is repeated until some good playing strategies have evolved. Such strategies have been shown to beat human experts. The game in Chellapilla and Fogel's case is not chess but checkers, or draughts as it is called in Britain. The authors point forcefully to the fact that the evolutionary process actually creates its own intelligence rather than relying on that of a programmer. Referring to the method that eventually defeated Kasparov, they write: "Every 'item' of knowledge was pre-programmed. In some cases the programs were even tuned to defeat particular opponents..."

Although neural networks were first suggested in the early 1940s, it is only in the past 15 years or so that they have been used with some success as systems that can learn to recognize patterns in a variety of scientific and industrial applications. Conventionally they consist of layers of cells (artificial neurons) where interconnections between neurons in adjoining layers are made through 'weights', while the body of the cell performs summations that cause it to 'fire' at an output (the axon) when the incoming weighted activity overcomes some mathematically specified gradient function. The weights are thought to resemble the action of synapses in real neuronal cells found in biological brains, where neurons connect and learning takes place through the alteration of such weights.

So an artificial network (which can have one of many popular topologies) learns to approximate an input-output function by being given examples of the in- and out-values of such a function. It does this through one of a variety of weight-adjustment procedures known as learning algorithms. The learned tasks can be as esoteric as recognizing faces where it would be hard for a programmer to work out *a priori* a function that would distinguish between such patterns. However the techniques used by Chellapilla and Fogel do not make use of learning algorithms such as these, but arrive at suitable synaptic-weight functions through an evolutionary process.

They use a population of 15 neural networks with randomly selected weights and the 'offspring' of such parent networks containing a variation of the parents' weights. The task for the network is to evaluate board images, while the playing proceeds along Shannon's minimax lines. All parents and offspring play five games against opponents randomly selected from their midst and are awarded points based on their successes and failures. The 15 most successful networks are then chosen to be the new generation, offspring are produced and the process repeats. One hundred generations take about two days of computing on a 400 MHz Pentium machine. After 250 generations the best network is chosen to play human all-comers on the Internet, who in turn are evaluated in terms of the excellence of their playing on a national categorization. The network was able to achieve a top categorization (that is, 'A') on this scale, to defeat two 'expert' category players and to draw with a 'master'.

Chellapilla and Fogel stress that evolutionary methods can develop machine intelligence without being programmed, and



100 YEARS AGO

During two or three visits to Paris and Nice some years ago, I discussed with many French astronomers, whom I was privileged to count among my personal friends, the question of the large telescopes of the future. Among the conclusions come to, the first was that the glass industry was not in a position to grapple with astronomical requirements, and hence when reflectors of 8 or 10 feet diameter were talked of it was understood that they must be made of porcelain with a glass surface. ... During the last few years we have heard a great deal of an enormous telescope to be constructed on the occasion of the Paris Exhibition of 1900; a reflector of 10 feet aperture, such as was discussed in 1875, but it would seem that now as then the glass industry is not able to furnish a disc of this size, for after all it has been determined to construct a refractor, and mount it as I suggested nearly twenty years ago in front of a siderostat. ... It is the intention of the Syndicate to erect in connection with this telescope a Palais de l'Optique near the Eiffel Tower, containing a hall capable of holding some 4000 persons, and in fine weather images of the various celestial bodies are to be thrown on a screen 20 metres in the side by means of secondary magnifiers. Thus an image of the moon 16 metres in diameter, and of Mars 3.70 metres in diameter, are promised to the abonnés. From *Nature* 21 December 1899.

50 YEARS AGO

Only two large mammals of South Africa have become extinct in the historical period, namely, the quagga and the blue buck. The blue buck (*Hippotragus leucophoeus*) lived in the south-west part of Cape Colony, and is believed to have been exterminated about 1799. About half a dozen mounted specimens have been preserved, mostly rather bad, and perhaps about three more pairs of horns exist the determination of which is doubtful. No specimen of the skull is known to exist, except, perhaps in some of the mounted specimens. On recently visiting the Hunterian Museum (Zoological Section) at the University of Glasgow, I saw a specimen that at once struck me as probably the skull of the extinct blue buck. There is no history attached to the specimen; but it is believed to have belonged to the Hunterian Collection. As William Hunter died in 1783, the specimen could date back to about the middle of the eighteenth century. From *Nature* 24 December 1949.

demonstrate¹ some of the generality of this principle in other games such as tic-tac-toe (noughts and crosses) and those involving a mix of collaborative strategies between the players (the so-called Prisoner's Dilemma). All of this shows that organisms in nature achieve what is called intelligence through a fascinating mix of evolution, adaptation and learning — with the possibility of inspiring those interested in computation not only to build smarter machines but also to get a

better understanding of the nature of intelligence itself. ■

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Diabetes

Insulin resistance and obesity

Michael W. Schwartz and Steven E. Kahn

Type 2 diabetes mellitus is a serious health problem in the Western world. It arises when resistance to the glucose-lowering effects of insulin combines with impaired insulin secretion to raise the levels of glucose in the blood beyond the normal range. Studies into the molecular basis of insulin resistance have focused on the peroxisome proliferator-activated receptor gamma (PPAR γ). This molecule, a member of the nuclear-hormone-receptor family, is the cellular target of thiazolidinedione drugs, which are used to treat diabetes by increasing sensitivity to insulin.

What are the endogenous ligands for PPAR γ ? How does it promote the insulin-stimulated uptake of glucose? And is this effect essential for the normal action of insulin? The answer to the last of these questions may be nearer thanks to a study by Barroso *et al.*¹ on page 880 of this issue. They

report the identification of two loss-of-function mutations of PPAR γ that are associated with severe insulin resistance and type 2 diabetes mellitus in humans. Although such mutations are rare — detected in just three of 85 insulin-resistant people, and none of 314 controls — the implication that PPAR γ is required for normal insulin sensitivity in humans is an important advance.

Found in the nucleus of many cells, particularly fat cells, PPAR γ is both a receptor and a transcription factor. When PPAR γ is bound by ligand, such as a thiazolidinedione, it becomes activated and binds to specific DNA sequences in gene promoters. Then, in complex with another transcription factor known as the retinoid X receptor (RXR), it activates the transcription of specific genes² (Fig. 1, overleaf). One of the best-studied effects of activated PPAR γ is its ability to induce differentiation of fibroblasts or other undifferenti-

ated cells into mature fat cells². Signalling by the PPAR γ -RXR complex is also implicated in the synthesis of biologically active compounds by vascular endothelial cells³ and circulating immune cells⁴. Mutations in PPAR γ may contribute to cancer⁵, and increased PPAR γ signalling (owing to a mutation that increases its intrinsic activity) is also associated with human obesity⁶.

Barroso *et al.*¹ now show that the people affected by loss-of-function PPAR γ mutations (one affects a mother and her son; the other affects an unrelated woman) share common elements of the 'insulin resistance syndrome'. Symptoms include insulin resistance, diabetes, high blood pressure, dyslipidaemia (an abnormal plasma-lipid profile) and a skin-pigmentation disorder known as acanthosis nigricans. But a cardinal feature of the insulin-resistance syndrome that these people do not show is obesity. Reduced PPAR γ signalling therefore seems to cause insulin resistance in the absence of obesity.

This observation contrasts sharply with the symptoms of gain-of-function mutation of PPAR γ , reported last year by Ristow *et al.*⁶. In their study, obesity was associated with relatively low levels of insulin, suggesting an *increased* sensitivity to insulin. However, neither report^{1,6} includes a measurement of insulin sensitivity. Moreover, a third mutation in PPAR γ has variable effects on body weight and insulin sensitivity^{7,8}. Nevertheless, all of these findings indicate that by increasing PPAR γ function it may be possible to prevent insulin resistance from occurring when normally it would (for example, in the obese state). Conversely, mutations in PPAR γ that cause reduced function could lead to insulin resistance in lean people, in whom it would not normally occur.

Antarctica

Vast snow dunes frozen in time

Release of data gathered during the Cold War continues to deliver scientific surprises. The latest example emerged at last week's American Geophysical Union meeting in San Francisco, where glaciologists reported new findings about Antarctica based on satellite data. The revelation came from comparisons of modern satellite images of snow dunes (such as the one shown here) with recently declassified pictures originally taken by intelligence satellites in 1963.

The first complete map of Antarctica, produced in 1997 with data from the Canadian satellite Radarsat, revealed many unexpected features, including vast tracts of snow dunes. These

megadunes are up to 100 kilometres long, lie 1 or 2 kilometres apart but are only a few metres high. In East Antarctica fields of the dunes cover an area larger than the state of California.

At the meeting Mark Fahnestock (University of Maryland) described how he and Ted Scambos (University of Colorado) compared data from satellites of the US National Oceanic and Atmospheric Administration and from the 1960s military images. It might be thought that the snow dunes would move, even if only slowly, because of the fierce, constant winds that blow across the East Antarctic plateau. It turns out, however, that they have not — at least over the past 30 years. Little is



known about how the megadunes formed, but it is unlikely that they grew from drifting snow in the way that sand dunes are built from sand.

Across the other side of the continent, on the West Antarctic ice sheet, vast streams of ice flow into the sea. The source of the ice streams has so far eluded explorers and satellites alike. At another talk at the meeting, Robert Bindshadler

(NASA Goddard Space Flight Center) described high-resolution data from Radarsat that reveals several small and slow-flowing tributaries that are feeding the large frozen rivers with snow from the interior. One of the biggest uncertainties in predicting sea-level rises in response to climate change is the uncertain behaviour of the Antarctic ice sheets. So new data (however old) is always welcome. **Sarah Tomlin**

NASA GODDARD SPACE FLIGHT CENTER

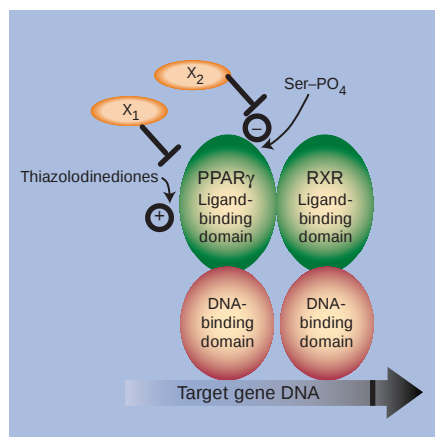


Figure 1 Molecular complex formed by dimerization of PPAR γ and the retinoid X receptor (RXR). Binding of ligand such as thiazolidinedione to either transcription factor can activate the complex, allowing the DNA-binding domain to bind the promoter region of target genes and activate transcription. Barroso *et al.*¹ have shown that mutations that impair ligand binding (X_1) disrupt this process and are associated with insulin resistance and normal body weight in humans. Serine phosphorylation (Ser- PO_4) of PPAR γ inhibits its activity, and mutation of an adjacent amino acid (X_2) blocks this site. This increases PPAR γ signalling, and is associated with obesity⁶.

Although disease-causing mutations of PPAR γ are rare, might the insulin resistance associated with human obesity result from impaired PPAR γ signalling in the absence of a mutation? Insulin resistance is especially likely to occur when excess fat is deposited within the abdominal cavity. This reduces the insulin sensitivity of fat cells and also of other tissues including skeletal muscle and liver. But how might expanding adipose stores causes insulin resistance? One explanation is that increased release of free fatty acids from triglyceride-laden fat cells provides an alternative metabolic substrate, which decreases the need for glucose as a fuel. As a result, insulin-stimulated glucose clearance from the blood is reduced, an effect that is manifest as insulin resistance.

However, because some free fatty acids may be PPAR γ ligands², an alternative explanation presents itself. If obesity alters the availability of these fatty acids, it could reduce PPAR γ signalling and produce insulin resistance. But there are other ways to regulate the function of PPAR γ . For example, phosphorylation of PPAR γ on serine residues reduces its function, even in the presence of thiazolidinediones^{2,9}. This is another potential mechanism whereby expanding fat stores might impair PPAR γ function.

The two PPAR γ mutations reported by Barroso *et al.*¹ lead to amino-acid substitutions in regions of the molecule involved in ligand binding. As a result, these changes

impair the activation of PPAR γ by thiazolidinediones. By contrast, the obesity-inducing PPAR γ mutation reported by Ristow *et al.*⁶ results in an amino-acid substitution adjacent to the serine phosphorylation site. This mutation impairs phosphorylation, thereby increasing PPAR γ function. In each case, affected patients have one mutant and one normal allele, suggesting that the mutant PPAR γ molecule dominates functionally over the normal protein. Indeed, Barroso and colleagues found that the function of normal PPAR γ was impaired when they co-expressed it in tissue culture with either of the mutant proteins. Such 'dominant-negative' mutations are well documented in other nuclear-receptor systems, and they help to explain how a single mutant allele can cause disease.

The study of PPAR γ mutations is expanding what we know about the involvement of this molecule in human health and disease. However, the demonstration of clinical abnormalities in a small number of patients who have a mutation is not proof that the mutation caused the symptoms. Determining how these patients respond to treatment with thiazolidinediones would provide important additional information. Moreover, neither insulin sensitivity nor insulin secretion were quantified in people with PPAR γ mutations, yet the interaction of these two parameters is critical for glucose homeostasis¹⁰. The importance of taking these measurements is highlighted by the presence of type 2 diabetes in three of four obese people with the gain-of-function PPAR γ mutation⁶. Perhaps the low insulin levels in these people reflect impaired insulin secretion rather than increased insulin sensitivity.

We need more information before we can conclude that too much PPAR γ causes obesity without the expected metabolic consequences, whereas too little PPAR γ elicits the metabolic consequences without obesity. But continued study of this important molecule could yield new approaches to the treatment of diseases such as obesity and diabetes, which take an enormous toll on human health. ■

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Daedalus

The art of slow change

Art, says Daedalus, seems to divide into two main camps. Some arts are quite static (pictures, statues and so on). Others (music, cinema, ballet) supply rapid change for a short time. But our aesthetic sense evolved in a world of constant slight change. Much of the charm of the natural world, such as the sea, the sky, the landscape, and indeed the appreciation of human fellowship, depends on slow change within certain expected limits. Daedalus is now exploring this neglected aesthetic.

The only current art form of slow change, gardening, gives constant pleasure from the steady subtle development of the plants. It is remarkably popular. But no engineering structures are designed to grow like plants. Instead, our buildings and monuments, though static, usually aim to be 'new and exciting'. This is self-defeating; shock and excitement are the most fleeting, least sensible goals for an architect or mason. Yet a building which changed slowly all the time would pose daunting technical challenges. So as a pilot project, Daedalus is devising a slowly-changing statue. Its pose and demeanour will drift subtly all the time. Instead of rapidly fading into the unnoticed urban background, it will retain interest and value to the frequent viewer.

Similarly, modern display technology should make possible a slowly-changing picture, perhaps like the ageing portrait of Dorian Gray in Oscar Wilde's story. It would drift slowly and subtly on a timescale of hours, days or months. It might play out a slow story, drift seemingly at random, or follow some environmental lead; but every time one glanced at it, it would be subtly different.

The most pleasing forms of change will take a long time to recognize and optimize — existing art forms have taken centuries to reach their current state of impasse. But in utilitarian mood, Daedalus likes the idea of driving his pictures or statues from the weather forecast, or the stock-market index. The viewer might come to think — or at least to intuit — 'The mayor looks happy today, it's going to be sunny' or 'Keynes has had an angry slouch all week, maybe it's time to shift a little into gilts'. Municipal patrons of the arts will support DREADCO's project more open-handedly if it serves a practical and civic-minded purpose.

David Jones

The further Inventions of Daedalus (Oxford University Press), 148 past Daedalus columns expanded and illustrated, is now on sale. Special *Nature* offer: m.curtis@nature.com

A funerary feast fit for King Midas

A royal banquet has been reconstructed from residues in pots found inside the tomb.

We have chemically analysed the ancient organic contents of vessels from the Tumulus MM, 'Midas Mound'¹, the site at Gordion in central Turkey that is the likely tomb of King Midas. The analysis revealed that a spicy meal of sheep or goat and pulses was eaten by mourners at a feast before the interment. We also identified a mixed fermented beverage of grape wine, barley beer and honey mead in the most comprehensive Iron Age drinking set ever found, comprising numerous bronze mixing and serving vessels and more than 100 bowls. Besides providing direct and dramatic evidence for ancient Mediterranean cuisine and customs, our findings have an important bearing on the cultural antecedents of Midas's Phrygian kingdom and on the wider application of molecular archaeological techniques to other ancient foods and beverages.

Preservation conditions were extraordinarily good inside the tomb, which is the earliest known intact wooden structure in the world, dated at about 700 BC. The body of a male, aged 60–65, was laid out in state on a thick pile of dyed textiles in a unique log coffin². The identification of the body as King Midas is strongly supported by the monumental size of the earthen mound built over the tomb, the richness of the burial goods, and the contemporaneous Assyrian inscriptions. The coffin and 14 pieces of fine wooden furniture³ had been placed in the tomb after being used in the ceremonies.

Our chemical reconstruction (Fig. 1) of the banquet entrée is based on well preserved 'fingerprint' compounds. Triacylglycerols, composed principally of saturated palmitic (C_{16}) and stearic (C_{18}) fatty acids, with small amounts of unsaturated oleic ($C_{18:1}$) fatty acids, predominate in the residues. These compounds, together with cholesterol and the C_6 , C_8 , C_{10} saturated acids (caproic, caprylic and capric acids, respectively) can best be explained as deriving from sheep or goat fat. A rancid odour, which may have come from this fat, was detected by the excavators when the tomb was opened. Other compounds indicate that the meat was first barbecued before being cut off the bone and seasoned with Mediterranean herbs and spices.

The major constituents of the mixed fermented beverage are tartaric acid and its salts (occurring naturally in large amounts only in grape and its products, including wine⁴), calcium oxalate ('beerstone', the main precipitate of barley beer⁵) and

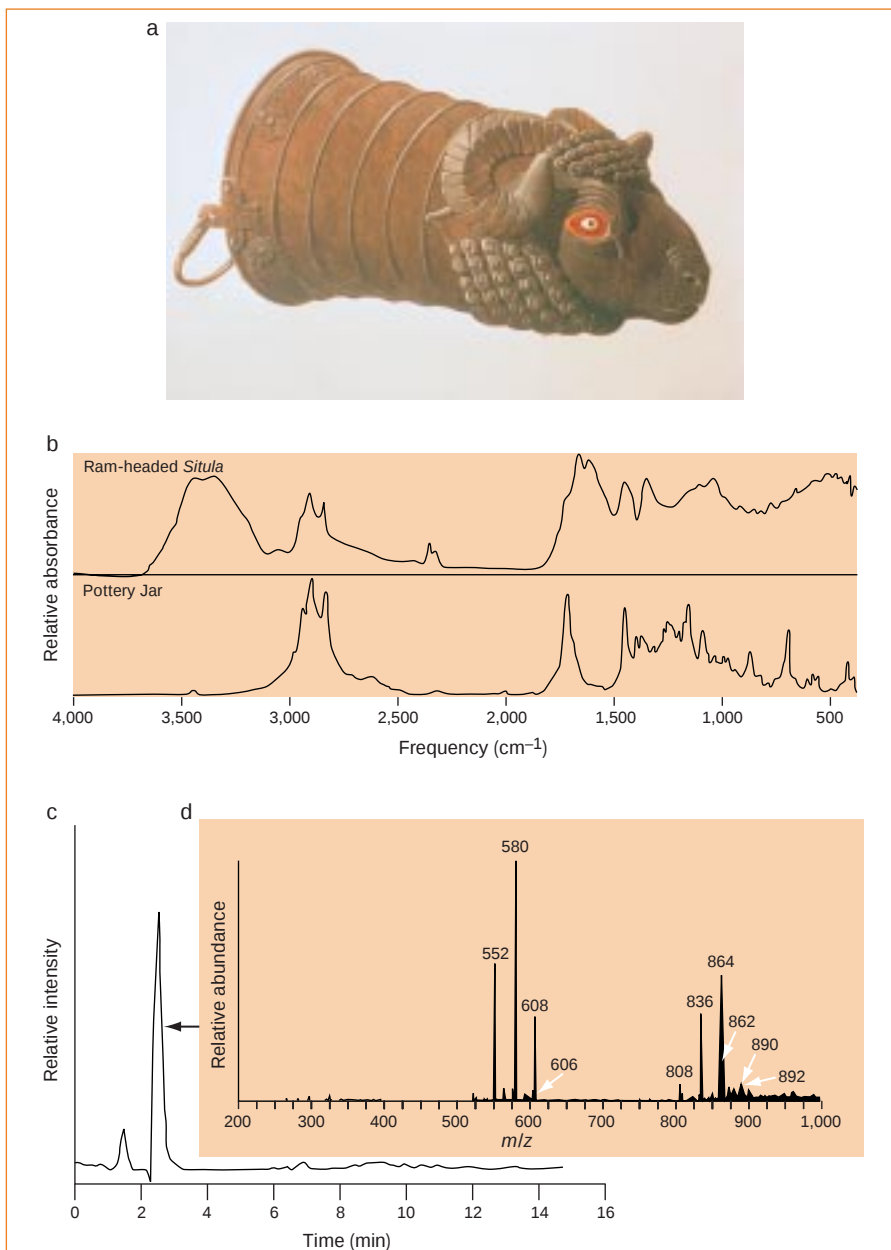


Figure 1 Analysis of vessel contents in the 'King Midas' tomb. **a**, Bronze ram-headed *situla*. **b**, Complementary analyses of the contents of the *situla* (purple line) and food remains from inside a pottery jar (red line). Diffuse-reflectance Fourier-transform infrared spectra typify the methanol extracts of 16 beverage and 14 food samples. The *situla*'s residue is characterized by strong hydrocarbon bands at 2,930/2,860, 1,460, 1,360 and 720 cm⁻¹, best explained by long-chain esters of beeswax. Carboxylate absorptions between 1,670 and 1,610 cm⁻¹ and between 1,610 and 1,570 cm⁻¹ correlate with calcium oxalate ('beerstone') and calcium tartrate, respectively. A broad band at 3,450–3,400 cm⁻¹ is due to hydroxyl groups. The food residue, by contrast, lacks carboxylate and hydroxyl absorption, and has a marked carbonyl doublet at 1,750–1,730 cm⁻¹. Its hydrocarbon bands have better definition in the 'fingerprint' region at 1,420, 1,390, 1,170, and 1,120 cm⁻¹. These data would best be accounted for by lamb fat. **c**, High-performance liquid chromatogram (HPLC) of a chloroform-methanol extract of the food residue shows that triacylglycerols account for the peak at a retention time of 2.55 min (total ions) representing 90% of the lipid fraction and about 10% of the ancient food residue. **d**, Its mass spectrum is dominated by protonated palmitodistearin (m/z 864), with lesser amounts of dipalmito-stearin (836) and tripalmitin (808). Small peaks at 862, 892 and 890, respectively, are due to oleo-palmito-stearin, tristearin and 2-oleodistearin, the latter being prevalent in pulses. The diacylglycerides at 608, 606, 580 and 552 are fragmentation products. Other components of the food samples were identified by gas chromatography–mass spectrometry, sometimes preceded by direct thermal extraction. These include: anisic acid (from anise or fennel), chondrillasterol (lentil), elaidic acid, the *trans* isomer of oleic acid (olive oil), a range of polycyclic aromatic hydrocarbons, including phenanthrene, and alkyl phenol derivatives such as cresol (barbecued meat), and α -terpineol and terpenoid compounds (spices).

beeswax (a group of marker compounds⁶ that are not easily filtered out from mead).

The Homeric epics^{7,8}, reflecting both Greek and Anatolian traditions of the eighth century BC and earlier, describe outdoor funeral banquets in which skewered and roast sheep and goat were served, together with a mixed fermented beverage (Greek *kykeon*)⁹ similar to that in the Midas tomb. (Barley grains were added to *kykeon*, which may have been in the form of beer.) This beverage, in which other fruits such as apple and cranberry might have been used instead of grapes, had long been a traditional drink in Europe¹⁰, suggesting that the Phrygian population could have been of European extraction, perhaps from the Balkans or northern Greece.

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Game theory

Losing strategies can win by Parrondo's paradox

In a game of chess, pieces can sometimes be sacrificed in order to win the overall game. Similarly, engineers know that two unstable systems, if combined in the right way, can paradoxically become stable. But can two losing gambling games be set up such that, when they are played one after the other, they becoming winning? The answer is yes. This is a striking new result in game theory called Parrondo's paradox, after its discoverer, Juan Parrondo^{1,2}. Here we model this behaviour as a flashing ratchet³, in which

winning results if play alternates randomly between two games.

There are actually many ways to construct such gambling scenarios, the simplest of which uses three biased coins (Fig. 1a). Game A consists of tossing a biased coin (coin 1) that has a probability (p_1) of winning of less than half, so it is a losing game. Let $p_1 = 1/2 - \epsilon$, where ϵ , the bias, can be any small number, say 0.005.

Game B (Fig. 1a) consists of playing with two biased coins. The rule is that we play coin 2 if our capital is a multiple of an integer M and play coin 3 if it is not. The value of M is not important, but for simplicity let us say that $M = 3$. This means that, on average, coin 3 would be played a

little more often than coin 2. If we assign a poor probability of winning to coin 2, such as $p_2 = 1/10 - \epsilon$, then this would outweigh the better coin 3 with $p_3 = 3/4 - \epsilon$, making game B a losing game overall.

Thus both A and B are losing games, as can be seen in Fig. 1b, where the two lower lines indicate declining capital. If we play two games of A followed by two of B and so on, this periodic switching results in the upper line in Fig. 1b, showing a rapid increase in capital — this is Parrondo's paradox. What is even more remarkable is that when games A and B are played randomly, with no order in the sequence, this still produces a winning expectation (Fig. 1b).

This phenomenon was recently proved mathematically¹ for a generalized M and analysed in terms of entropy based on Shannon's information theory³. We used the flashing brownian ratchet⁴ to explain the game by analogy. The flashing ratchet can be visualized as an uphill slope that switches back and forth between a linear and a sawtooth-shaped profile. Brownian particles on a flat or sawtooth slope always drift downwards, as expected. However, if we flash between the flat and saw-tooth slope, the particles are 'massaged' uphill. This is only possible if the sawtooth shape is asymmetrical in a way that favours particles spilling over a higher tooth.

The flat slope is like game A, where the bias ϵ is like the steepness of the slope. Game B is like the sawtooth slope, where the difference between coin 2 and coin 3 is like the asymmetry in the tooth shape. In the brownian ratchet case, there are two types of slope, with falling particles, but when they are switched the particles go uphill. Similarly, two of Parrondo's games have declining capital that increases if the games are switched or alternated. The games can be thought of as being a discrete ratchet and are known collectively as a parrondian ratchet.

Game theory is linked to various disciplines such as economics and social dynamics, so the development of parrondian-like strategies may be useful, for example for modelling cases in which declining birth and death processes combine in a beneficial way.

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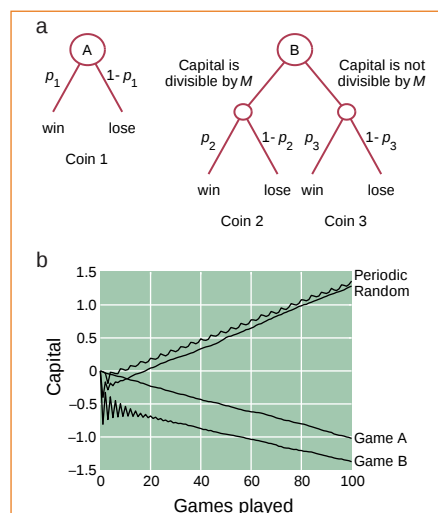


Figure 1 Game rules and simulation. **a**, An example of two games, consisting of only three biased coins, which demonstrate Parrondo's paradox, where p_1 , p_2 and p_3 are the probabilities of winning for the individual coins. For game A, if $\epsilon = 0.005$ and $p_1 = 1/2 - \epsilon$, then it is a losing game. For game B, if $p_2 = 1/10 - \epsilon$, $p_3 = 3/4 - \epsilon$ and $M = 3$ then we end up with coin 3 more often than coin 2. But coin 3 has a poor probability of winning, so B is a losing game. The paradox is that playing games A and B in any sequence leads to a win. **b**, The progress of playing games A and B individually and when switching between them. The simulation was performed by playing game A twice and game B twice, and so on, until 100 games were played; this is indicated by the line labelled 'Periodic'. Randomly switched games result in the line labelled 'Random'. The results were averaged from 50,000 trials with $\epsilon = 0.005$.

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Enhanced yield of photoinduced electrons in doped silver halide crystals

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The conventional photographic process^{1–3} involves several steps: the photogeneration of electron–hole pairs in crystals of a silver halide; the reduction of silver cations to atoms by some fraction of these electrons; the subsequent build up of atoms to give clusters (the ‘latent image’); and the complete reduction by a developer of crystallites having more than a critical number of silver atoms per cluster. The effective quantum yield, Φ_{eff} , of photoinduced electron–hole pairs produced per photon absorbed is less than the theoretical limit ($\Phi_{\text{theor}} = 1$), because of the fast recombination of some fraction of the pairs^{1–6}. Here we describe an approach for enhancing the yield of useful photogenerated electrons, in which the silver halide is doped with formate ions, HCO_2^- . The dopant ions act as hole scavengers, thus enhancing the escape of electrons from pair recombination. Moreover, the resulting $\text{CO}_2^{\cdot -}$ radical can itself transfer an electron to another silver cation, so raising the theoretical yield to two silver atoms per photon absorbed. This photoinduced bielectronic transfer mechanism is strictly proportional to the light quanta absorbed—the dopant ions do not induce spontaneous reduction of silver cations in the dark—and appears to be close to the theoretical limit of efficiency. The efficiency is constant at all illumination levels and applies to both dye-sensitized and unsensitized crystals. We suggest that this approach is a promising route for improving the performance of photographic emulsions⁷.

According to the classical model of photography^{1–3}, at least some of the initially photogenerated electrons reduce silver cations to atoms. A cation near one of these atoms is then reduced by another electron, and so on; the result is a cluster of silver atoms. Some other (initially photogenerated) electrons are lost by direct recombination with holes, with (at low temperatures) the emission of light. Before being irreversibly trapped, holes are also able to oxidize the newly formed silver atoms, so counterbalancing somewhat the former reduction. The ensemble of photoinduced clusters constitutes the latent image. A critical number n_c of silver atoms per cluster must be formed to induce (in a second step) the complete reduction of the crystallite by a developer.

Different strategies^{1–5} are used to produce—at a given flux of light and crystal cross-section—the required n_c with the shortest exposure, and thus to enhance the sensitivity of the emulsion containing the silver halide crystals. One quite efficient approach is to reduce the fraction of electron–hole pairs that recombine by enhancing transient electron trapping in specific surface sites (sulphide or/and gold centres). Classical developers then require only $n_c = 3$ atoms per crystallite⁸. The initial number n_i of photons absorbed to reach this threshold (quantum sensitivity) is generally from 10 to 30 per crystal⁶, the difference ($n_i - n_c$) being lost by recombination. Thus the effective quantum yield $\Phi_{\text{eff}} = n_c/n_i$ is in the range 0.30–0.10. Values of $n_i = 6$ –8 ($\Phi_{\text{eff}} = 0.50$ –0.37) have been reported at optimized S–Au sensitization of octahedral crystallites, after correction for fogging level^{6,7}, but these figures are much lower than $\Phi_{\text{theor}} = 1$.

Another possible method of enhancing emulsion sensitivity involves scavenging the holes by adding an electron donor (reduction sensitizer) before exposure^{4–6}. Ideally, pre-reduction of these emulsions yields a dimer (Ag_2) per crystal. It is supposed to scavenge

holes, and to yield the equivalent of two electrons for silver cluster formation (one electron escaping from the recombination with the hole scavenged by Ag_2 , the second coming from $\text{Ag}_2^{\cdot +}$)^{4–6}. Therefore a third atom only has to be provided by light to reach n_c . Indeed, the value of n_i is found now to be 2–3 photons per crystal⁶ (in that case, $\Phi_{\text{eff}} = n_c - 2/n_i = 0.50$ –0.33). However, the limit of 2 pre-reduced atoms per crystal is easily exceeded in practice, and as much as 50% of the crystal population may be developable, irrespective of the number of photons absorbed (the fogging effect).

Here we report the effect of doping the silver halide crystals with the bielectronic donor ion formate (HCO_2^-), which by itself is not a direct reduction sensitizer (the redox potential $E^\circ(\text{CO}_2^{\cdot -}/\text{HCO}_2^-) = 1.07 \text{ V}_{\text{NHE}}$ at pH 7 in water) and is unable to reduce silver cations in the dark, so avoiding the risk of fogging. In contrast, the formate ion can scavenge photo-produced holes, and moreover, the radical product of such scavenging ($\text{CO}_2^{\cdot -}$) is a strong electron donor able to inject a supplementary electron into the conduction band to form—from a silver ion associated with an atom (Ag^+) or a cluster (Ag_n^+)—a supplementary silver atom: $E^\circ(\text{CO}_2/\text{CO}_2^{\cdot -}) = -1.9 \text{ V}_{\text{NHE}}$ in water⁹. Such behaviour of bielectronic donors is known in the interaction of radiation and of light with aqueous solutions (molecular hydrogen, formate, alcohols)^{9–12}, and the photoelectrochemistry that occurs at the interface of solutions and semiconductors^{13,14}. We prepared doped emulsions as described in Fig. 1 legend: both these and the reference emulsions were coated on a flat support, exposed to light at a wavelength λ_{exp} of

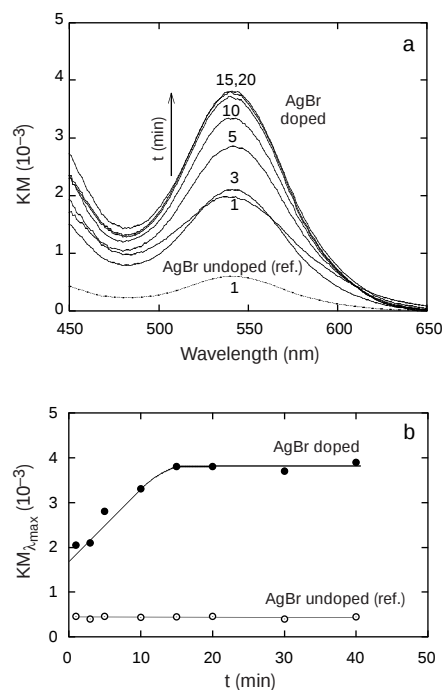


Figure 1 Effect of formate dopant on photoproduced silver clusters. **a**, Evolution with time of the absorbance spectra with 2 s illumination of formate-doped ($10^{-6} \text{ mol HCO}_2^-/\text{mol Ag}^+$) AgBr cubic grains (solid lines) and undoped AgBr cubic grains (dotted line), both sensitized by S–Au. The absorbance is derived from the Kubelka–Munk¹⁵ factor $\text{KM} = (1 - R)^2/2R$, where R is the diffuse reflectance. **b**, Time dependence of the KM factor at the absorption maximum for the undoped (open circles) and formate-doped (filled circles) emulsions. Formate doping of emulsions was performed during the precipitation of AgBr. Core cubic grains of AgBr (0.37 μm diameter), suspended in an aqueous gelatin solution, were first prepared using a controlled double jet of AgNO_3 and KBr solutions³. Then triple-jet precipitation was used to coat these grains with dopant HCO_2Ag as a 25-nm-thick shell, so that the total grain diameter was 0.42 μm . The overall relative molar concentration $\text{HCO}_2^-/\text{Ag}^+$ was 10^{-7} – 10^{-5} . The emulsions, consisting of colloidal suspensions of AgBr microcrystals stabilized by gelatin, were then subjected to digestion for S–Au chemical sensitization and coated onto a flat support (1.5 g Ag^+ per m^2).

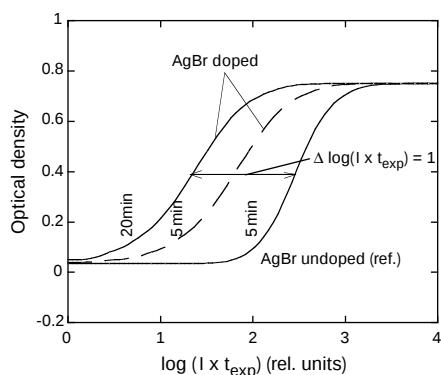


Figure 2 Sensitivity of undoped and formate-doped (10^{-6} mol HCOO^- /mol Ag^+) AgBr emulsions. Both emulsions were sensitized with S+Au. Development by M-AA-1 (aminophenol and ascorbic acid) for 8 minutes (ref. 8) was delayed by the indicated times, after a constant exposure time $t_{\text{exp}} = 10^{-2}$ s, at variable intensity I (in relative units).

410 nm, and studied by diffuse reflectance¹⁵, fluorometry, and sensitometry (absorbance measurement after photographic development).

In Fig. 1 we show the typical evolution with time of spectra which were obtained after exposure of doped and reference emulsions, and which we assigned to photoproducted silver clusters^{5,16}. The intensity at long times increases linearly with the dopant concentration until a plateau is reached at a ratio of 10^{-6} mol formate per mol Ag^+ . When in the dark, the formate-doped emulsion is completely stable. When illuminated (Fig. 1), its absorbance immediately after the exposure is five times that of the undoped emulsion; after 15 minutes, its absorbance is still twice that just after the exposure (10 times the reference absorbance).

In the presence of formate, the relative increase of absorbance with respect to the undoped emulsion within the exposure is assigned to hole scavenging by HCOO^- , which is faster than the electron-hole recombination or the trapping of unrecombined holes. Thus, more electrons escape from the pair recombination and are available to reduce silver cations to silver atoms; moreover, fewer silver atoms are oxidized by the holes.

Fluorescence at 77 K (due to electron-hole recombination) is detected in undoped emulsions exposed to light (fluorescence occurs at 560 nm if $\lambda_{\text{exp}} = 410$ nm; data not shown): this emission is completely quenched in formate-doped emulsions, confirming complete hole-scavenging by HCOO^- , at least for a concentration beyond 10^{-6} mol HCOO^- per mol Ag^+ .

In the range of minutes after the end of exposure of formate-doped emulsion, the number of silver atoms slowly increases until they have doubled (Fig. 1). In fact, the delayed supplementary formation of silver atoms arises from the second electron transfer to Ag^+ ions from the CO_2^- radical formed by hole scavenging during the exposure. The reaction is very slow, probably because the CO_2^- radical is trapped in the crystal matrix and because the direct reduction of free mobile silver ions would require a more negative redox potential^{17,18}. So we assign the slow supplementary silver atom formation to the reduction of Ag_2^+ or Ag_n^+ , possibly by electron hopping from CO_2^- into the conduction band. The maximum at 545 nm in these diffuse reflectance data with a rather long exposure is assigned to large clusters¹⁶. The absorbance is proportional both to the nominal concentration of atoms formed and to the extinction coefficient per atom. So the slow increase may arise from the formation of additional clusters, or from the growth of a few large clusters per crystal formed during the exposure and enriched by the new atoms. The first hypothesis is more probable if we consider the mechanism of the slow extra electron production in hydrogen-sensitized emulsions¹⁹.

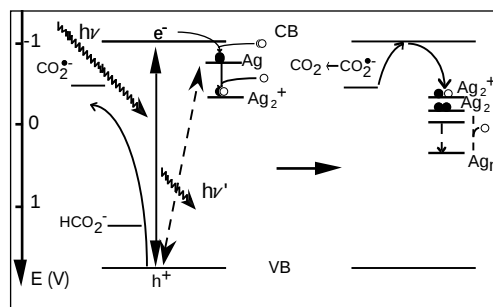


Figure 3 Diagram of the double action of formate in doped silver halides. The scale of energy levels refers to the normal hydrogen electrode potential (for AgBr, CB is -1.04 and VB at 1.6 V_{NHE}). Here CB indicates conduction band, and VB, valence band. The formate level is above the VB and HCOO^- scavenges the photo-hole. The energy level of the CO_2^- radical formed is such that an electron is transferred to charged silver dimers, yielding a second atom per photon absorbed. The energy levels of HCOO^- and CO_2^- in an AgBr matrix are shifted relative to those in solutions, due to the lack of solvation and to the matrix effect.

Quantitatively, as the absorbance at the plateau is just twice that immediately after the exposure, each CO_2^- radical formed by scavenging of a hole produces one supplementary silver atom in one of the clusters. Thus the overall mechanism implies extremely fast hole scavenging; this photoinduced bielectronic transfer is strictly proportional to the number of photons absorbed, down to the shortest exposure times that we studied.

The sensitivity of doped and undoped emulsions have been compared; this was done by measuring their absorbance after an exposure time t_{exp} of 10^{-2} s with a light source of variable intensity I and photographic development, which allows the investigation of effects caused by the absorption of only a few light quanta (Fig. 2). When the development is delayed 20 minutes after exposure, the number of photons required to induce development of half the grain population is 10 times less in doped than in undoped emulsions ($\Delta \log(I \times t_{\text{exp}}) = 1$). This enhancement in the sensitometric curves corresponds to the absorbance data and provides the same evidence of the double role of formate dopant as a hole scavenger and as a delayed secondary reduction source. We note that the sigmoid response curves of doped AgBr emulsions are systematically less steep than in undoped emulsions. Calculations by Silberstein²⁰ with small values of n_c predicted such a change in the curve shape; it arises from the Poisson statistics of the distribution throughout the crystal population of the photon number required for development⁶. It is remarkable that though the doping highly enhances the sensitivity, fogging remains at the same insignificant level.

We now consider absolute quantum yields, measured as in refs 6 and 8. We find that $n_i = 20 \pm 3$ photons per crystal must be absorbed in the undoped emulsions to make half of the grains developable ($\Phi_{\text{eff}} = n_c/n_i = 0.15 \pm 0.03$), in agreement with other data⁶. For formate-doped emulsions, we obtain 2 ± 0.3 photons per crystal. The resulting value $\Phi_{\text{eff}} = n_c/n_i = 1.5 \pm 0.3$ is exceptionally high, and approaches the theoretical limit of $\Phi_{\text{theor}} = 2$ suggested by the mechanism shown in Fig. 3 and confirmed by low-temperature fluorescence data. We note that as photon absorption is a quantum phenomenon, values of the threshold $n_c \geq 3$ cannot be reached by less than the integer number $n_i = 2$ photons per crystal (producing 4 silver atoms).

As most emulsions for practical use are also sensitized by dye molecules, in order to broaden their spectral absorption in the green and the red, our study was extended to the effect of formate-doping on dye-sensitized crystals. In such a system, light produces an excited state of the chromophore adsorbed at the AgBr surface; this excited state is known to inject an electron into the crystal conduction band. We therefore need to check whether a hole can

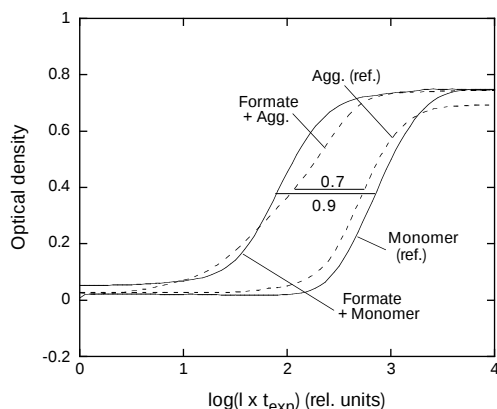


Figure 4 Effect of formate doping on the sensitivity of AgBr emulsions sensitized with carbocyanine dye. After the precipitation and doping (10^{-6} mol $\text{HCOO}^-/\text{mol Ag}^+$) by formate as in Fig. 2, the AgBr crystals were sensitized by the dye (3-(2-(3-(5-chloro-3-(3-sodium-sulpho-propyl)-benzoxazol-2-yl)-2-ethyl-allylidene)-5-chloro-benzoxazolium-3-yl)-propane-1-sulphonic acid betaine) in the monomeric form ($\lambda_{\text{max}} = 480$ and 500 nm, surface coverage = 0.2) and excited at $\lambda_{\text{exp}} \geq 477$ nm, or in the J-aggregate form (additional λ_{max} at 552 nm, surface coverage = 0.8) and excited at $\lambda_{\text{exp}} \geq 519$ nm. Development by M-AA-1 is delayed for 20 min, after $t_{\text{exp}} = 10^{-2}$ s.

also be transferred from the highest occupied molecular orbital (HOMO) of the dye to the Fermi level of the dopant formate, so blocking electron-hole recombination³. Our results from emulsions sensitized by a carbocyanine thought to fulfill the above criterion indicate that the sensitivity is indeed increased (Fig. 4), compared to emulsions without formate supposed to absorb the same quantity of photons. Depending on the surface coverage by dye molecules, these are in the form of either monomers or J-aggregates, which are two-dimensional arrays of edged-on adsorbed dye molecules with a large slip angle. The difference is about $\Delta \log(I \times t_{\text{exp}}) = 0.9$ for emulsions sensitized by dye monomers ($\lambda_{\text{max}} = 480$ and 500 nm), excited at $\lambda_{\text{exp}} \geq 477$ nm; for emulsions sensitized by J-aggregates (additional λ_{max} at 552 nm), excited at $\lambda_{\text{exp}} \geq 519$ nm, we obtain $\Delta \log(I \times t_{\text{exp}}) = 0.7$. This enables us to assess the effect of aggregates only without coexisting monomers. The sensitivity enhancement that is systematically observed in the presence of formate ion therefore attests to efficient hole transfer to the dopant formate from the HOMO level of the dye, in both the monomer and the J-aggregate forms. This mechanism is comparable with the scavenging of the intrinsic hole created by direct AgBr excitation, and results in a marked inhibition of recombination. □

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Periodic mesoporous organosilicas with organic groups inside the channel walls

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Surfactant-mediated synthesis methods have attracted much interest for the production of inorganic mesoporous materials, which can, on removal of the surfactant template, incorporate polymeric, organic, inorganic and organometallic 'guests' in their pores^{1,2}. These materials—initially made of silica^{3–5}, but now also available in the form of other oxides^{6–9}, sulphides^{10,11}, phosphates¹² and metals¹³—could find application in fields ranging from catalysis, adsorption and sensing technology to nanoelectronics. The extension of surfactant-mediated synthesis to produce inorganic-organic hybrid material (that is, materials that contain organic groups as an integral part of their framework structure) promises access to an even wider range of application possibilities. Such hybrid materials have been produced in the form of amorphous silicates (xerogels) that indeed display unique properties different to those of the individual components^{14–20}, but their random networks with broad pore-size distributions severely limit the shape and size selectivity of these materials. Mesoporous hybrid materials with periodic frameworks have been synthesized, but the organic groups are all terminally bonded to the pore surface, rather than incorporated into the pore walls^{21–26}. Here we describe a periodic mesoporous organosilica containing bridge-bonded ethene groups directly integrated into the silica framework. We are able to solvent-extract and ion-exchange the surfactant templates to create a stable and periodic mesoporous ethenesilica with high surface area and ethene groups that are readily accessible for chemical reaction. Recent syntheses of similar periodic mesoporous organosilicas^{27,28} and the ability to incorporate a variety of bridging organic and organometallic species raise the prospect of being able to fuse organic synthesis and inorganic materials chemistry to generate new materials with interesting chemical, mechanical electronic, optical and magnetic properties.

In a typical synthesis, a mixture of bis(triethoxysilyl)ethene (BTE) and tetraethylorthosilicate (TEOS) was added to a solution of cetyltrimethylammonium bromide (CTABr), NH_4OH and H_2O . Samples were prepared containing BTE:TEOS in mole fractions of 1:0 (BTE100), 0.75:0.25 (BTE75), 0.50:0.50 (BTE50), 0.25:0.75

(BTE25) and 0:1 (BTE0) based on Si contribution. After ageing for 4 days, the products were isolated and washed carefully with water. The surfactant was removed from the pores by mild solvent extraction or ion exchange that leave intact the mesopores and the framework. After a single extraction, the amount of surfactant collected was ~ 30 wt% of the initial material.

To prove that the ethene moiety was incorporated into the material as a bridge-bonded group, and that the materials were well-ordered and mesoporous, a series of samples was examined by powder X-ray diffraction, transmission electron microscopy (TEM), FT-Raman spectroscopy, ^1H magic angle spinning (MAS) NMR, ^{13}C and ^{29}Si cross-polarization (CP) MAS NMR spectroscopies, differential thermogravimetric analysis (DTGA), and N_2 gas adsorption (see Figs 5–11 in Supplementary Information).

Powder X-ray diffraction patterns for as-synthesized samples containing different proportions of BTE to TEOS are shown in Fig. 1a. Four peaks were observed for samples BTE50, BTE25 and BTE0, which could be indexed as the (100), (110), (200) and (210) reflections of a hexagonal symmetry lattice with a unit cell dimension $a \approx 47$ Å (see Figs 6 and 7 in Supplementary Information). Samples BTE100 and BTE75 showed the (100) diffraction peak, but the other three peaks were less intense. These results suggest the presence of a periodic arrangement of channels in a hexagonal geometry. We note that the intensities of the peaks become less intense as the relative amount of BTE to TEOS increases. As more SiO_2 is replaced with ethene groups, the electron density of the framework decreases relative to that of the surfactant-containing

pores. Moreover, removing surfactant from the pores increases the electron density of the framework relative to the pores²⁹, and increases the intensity of the diffraction pattern for all samples (Fig. 1b). Together these data demonstrate that ethene functionality has been incorporated into the framework of the periodic mesoporous silica.

TEM images of the materials are consistent with the powder X-ray diffraction results, showing hexagonal symmetry mesopores throughout the sample and a pore centre-to-centre distance of ~ 45 – 50 Å (Fig. 1c–e). The TEM image of as-synthesized BTE100 (Fig. 1c) shows that the material is as well ordered as BTE0 (MCM-41), even though a slight difference between their powder X-ray diffraction patterns was observed (see Fig. 8 in Supplementary Information). The quality of the hexagonal mesostructure is retained following a series of surfactant extractions (Fig. 1d). A TEM image of twice-extracted BTE100 viewed parallel to the channel axis is displayed in Fig. 1e.

Figure 2 shows DTGA traces of as-synthesized and solvent-extracted (once and twice) BTE100, and as-synthesized BTE0. A small mass loss of adsorbed water occurs in the range 40 – 80 °C. A weight loss of $\sim 30\%$ between 150 and 350 °C corresponds to surfactant and some ethene. ^{13}C CP-MAS NMR of a sample of BTE100, calcined in air at 350 and 400 °C for 6 hours, showed complete loss of surfactant and some ethene at 350 °C with complete removal of ethene by 400 °C. Variable-temperature powder X-ray diffraction (room temperature to $1,000$ °C) showed a decrease of ~ 10 Å in the d_{100} spacing of BTE100, roughly twice that of BTE0 despite similar initial unit-cell dimensions. This distinction probably

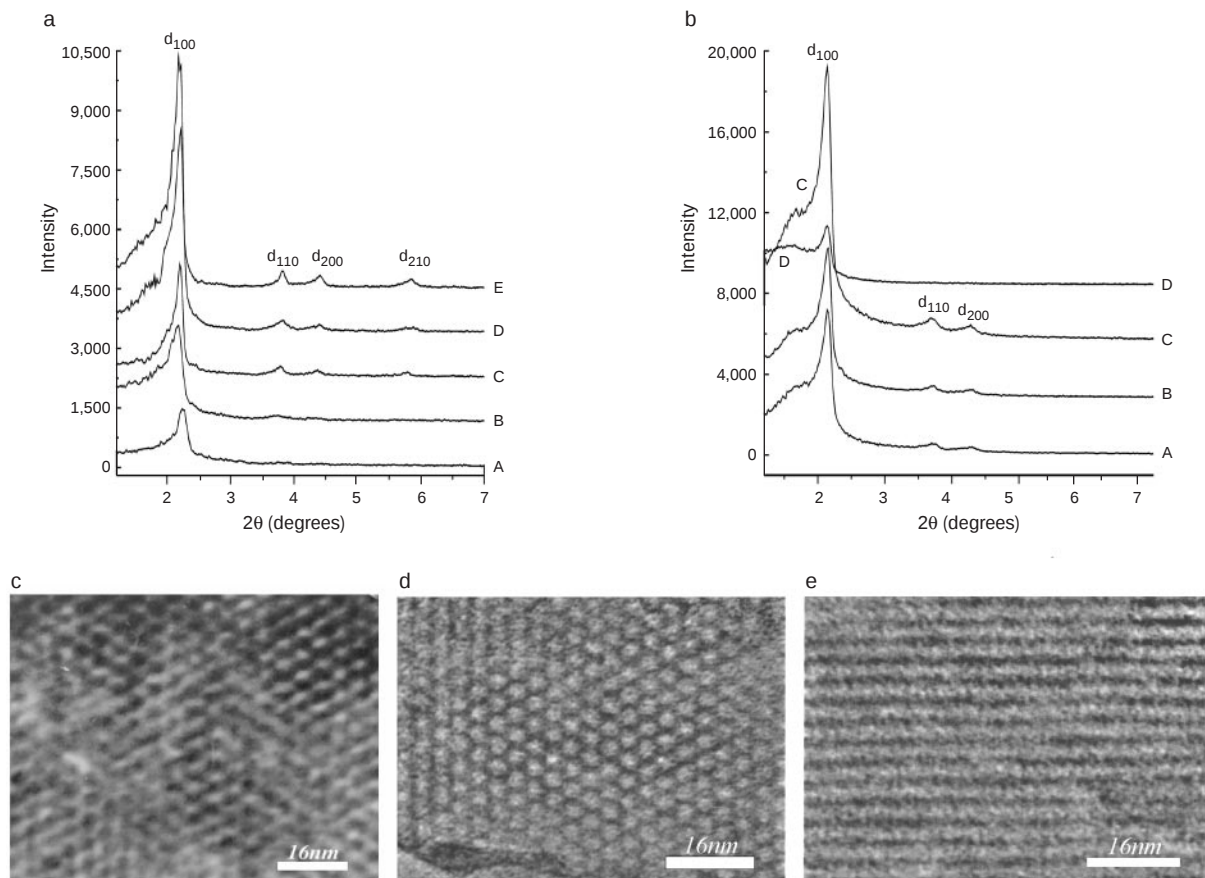


Figure 1 Powder X-ray diffraction patterns and TEM images of mesoporous ethenesilica. (See Figs 6–8 in Supplementary Information.) **a**, Powder X-ray diffraction (PXRD) patterns for as-synthesized samples containing different proportions of BTE and TEOS. Shown are the diffraction patterns for BTE100 (trace A), BTE75 (B), BTE50 (C), BTE25 (D) and BTE0 (E). The patterns confirm the hexagonal symmetry of the materials. **b**, Comparison of PXRD patterns of BTE100; after solvent-extraction (trace A), after ion-exchange (B), after additional solvent-extraction (C), and as-synthesized (D). The (110) and (200) reflections

are clearly visible after the surfactant was extracted. **c**, **d**, TEM images of the well-ordered hexagonal mesostructure of BTE100 as-synthesized and after two solvent-extractions, respectively. **e**, View parallel to the channel axis of the BTE100 sample (twice solvent-extracted). PXRD patterns were measured with a Siemens D5000 diffractometer using Ni-filtered $\text{Cu-K}\alpha$ radiation with $\lambda = 1.54178$ Å. TEM images were recorded on a Philips 430 microscope operating at an accelerating voltage of 100 kV.

arises from the 'elastic response' of the channels to loss of ethene from the silica walls. The mesostructure of BTE100 is maintained to over 1,000 °C, well after the ethene groups have been eliminated from the framework. This implies that the material 'healed' during the thermal elimination of the ethene moiety via condensation of silanol groups.

FT-Raman spectroscopy showed the presence of the ethene groups in as-synthesized and solvent-extracted samples (Fig. 3a, b). A spectrum for ion-exchanged BTE100 is shown in Fig. 3c. A C=C stretching mode at 1,580 cm⁻¹, absent in BTE0, remains after surfactant extraction. Its intensity increases with the amount of BTE in the framework. Bands around 2,800–3,200 cm⁻¹ and 1,300 cm⁻¹ are assigned to C–H stretching and C–H deformation modes of the surfactant and ethene groups.

Confirmation of incorporation of ethene groups directly inside the framework was obtained by NMR spectroscopy. The ¹H MAS NMR spectra of as-synthesized, solvent-extracted and ion-exchanged BTE100 samples are shown in Fig. 4a. Each sample shows a resonance at ~2 p.p.m. due to alkyl hydrogens of the surfactant, which decreases after surfactant extraction. Peaks at ~4 and 7 p.p.m. are attributed to silanol and ethene hydrogens, respectively. Upon solvent extraction, the ratio of silanol:ethene proton resonances increases, consistent with the replacement of cationic surfactants with protons to form silanol groups. ¹³C CP-MAS NMR shows a resonance at 144 p.p.m. attributed to ethene carbons, and resonances below 70 p.p.m. correspond to the carbons of the surfactant (Fig. 4b). An increase in ethene:surfactant intensity was observed after samples underwent solvent extraction and ion exchange.

²⁹Si CP-MAS NMR of as-synthesized samples show mostly T₃ (C=C)-Si-(OSi)₃ sites and some T₂(C=C)-Si-(OSi)₂(OH) sites at approximately -83 and -73 p.p.m., respectively (Fig. 4c). For comparison, Q₄ Si-(OSi)₄ and Q₃ (HO)-Si-(OSi)₃ sites of mesoporous silica (BTE0) were observed at -111 and -102 p.p.m., respectively. After solvent-extraction an increase in T₂, and the appearance of a small amount of T₁ (C=C)-Si-(OSi)(OH)₂ (about

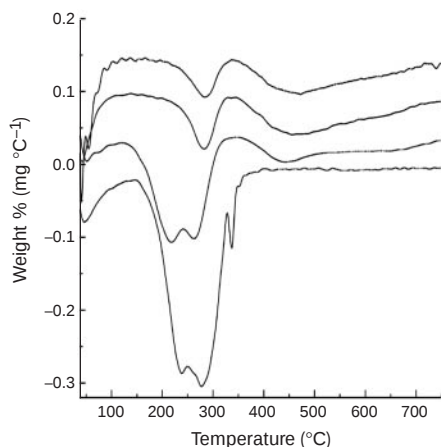


Figure 2 Differential thermogravimetric analysis (DTGA) of mesoporous ethenesilica. Traces a–c, results for BTE100; as-synthesized (trace a), once solvent-extracted (b) and twice solvent-extracted (c). Trace d, as-synthesized BTE0 (MCM-41). A transition around 80 °C corresponds to desorption of water from the channels. A second transition occurring between 150 and 250 °C corresponds to removal of most of the surfactant (space filling) in the material. A third transition occurring between 250 and 350 °C corresponds to the loss of the remaining surfactant (charge balancing) and the loss of some of the ethylene groups. Solid-state NMR of samples calcined at 400 °C did not show any trace of surfactant or ethylene groups, whereas samples calcined at 350 °C showed ethylene and trace amounts of surfactant. In samples containing BTE, a weight loss beyond 350 °C is attributed to loss of some ethylene groups and to loss of water, due to condensation of silanol groups. DTGA data were obtained at a heating rate of 5 °C min⁻¹ under a flow of N₂ on a Perkin-Elmer TGA7 instrument.

–64 p.p.m.) were observed as silanols replaced surfactants (see Figs 10, 11 in Supplementary Information). Minor Q₃ (–101 p.p.m.) and Q₄ (–111 p.p.m.) sites were seen after surfactant extraction. They probably arise from slight hydrolysis of ethene–silicon bonds^{30,31}. Spectra obtained without CP showed similar results.

Nitrogen adsorption isotherms (obtained at 77 K) for BTE100 materials that had been once, twice and thrice solvent-extracted and then vacuum thermally treated were all found to be type IV, which is characteristic of a mesoporous silica material. Analysis of the isotherms for the three samples yielded an average BET surface area of 637 m² g⁻¹, a pore volume of 0.60 cm³ g⁻¹ and a pore diameter of 39.4 Å (see Fig. 9 in Supplementary Information). With the unit-cell size of 47 Å, determined from powder X-ray diffraction, this yields a channel wall thickness of 7–8 Å.

To demonstrate the chemical accessibility of ethene groups, bromination of the ethene groups in BTE100 samples was attempted (see Fig. 5 in Supplementary Information). The sample

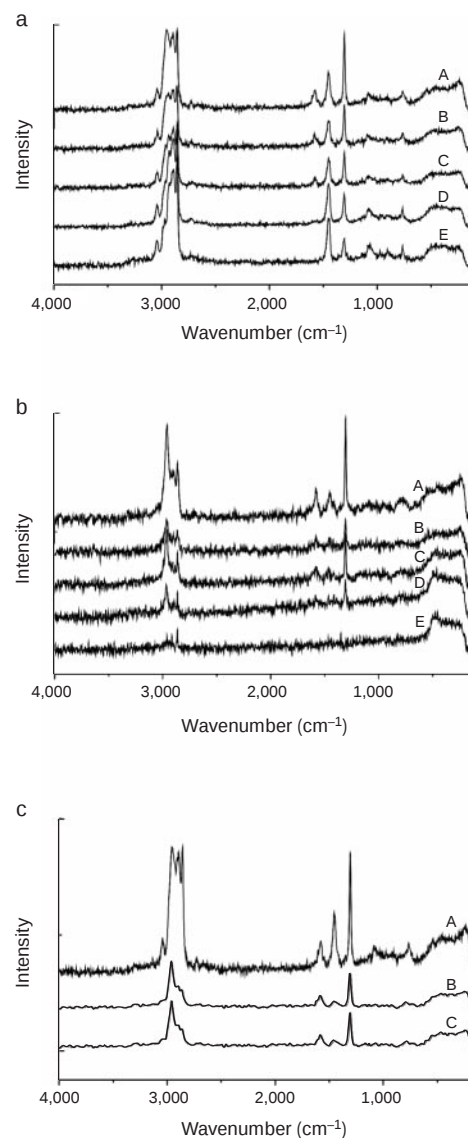


Figure 3 Raman spectra of mesoporous ethenesilica. **a, b**, As-synthesized and solvent-extracted samples, respectively. Each panel shows five spectra: trace A, BTE100; B, BTE75; C, BTE50; D, BTE25; E, BTE0. **c**, Spectra of BTE100: A, as-synthesized; B, after solvent-extraction; C, after ion-exchange. After solvent-extraction and ion exchange, all surfactant peaks disappeared while peaks assigned to ethylene groups remained. Raman spectra of the samples were collected using a Bomem MB-158 Fourier transform (FT) Raman spectrometer with an InGaAs near-IR detector and a Spectra Physics Diode Nd:YLF laser emitting at a wavelength of 1,064 nm and at a 350-kHz repetition rate.

was treated in refluxing $\text{CH}_2\text{Cl}_2/\text{Br}_2$ for 8 days. ^{13}C CP-MAS NMR of the product confirmed that the $\text{O}_3\text{Si}-\text{CH}=\text{CH}-\text{SiO}_3$ groups were entirely consumed during the bromination step. The peak originally observed at 147 p.p.m. was replaced with one at 12 p.p.m. In the ^{29}Si NMR spectrum, the T_2 and T_3 resonances at -73 and -83 p.p.m. for

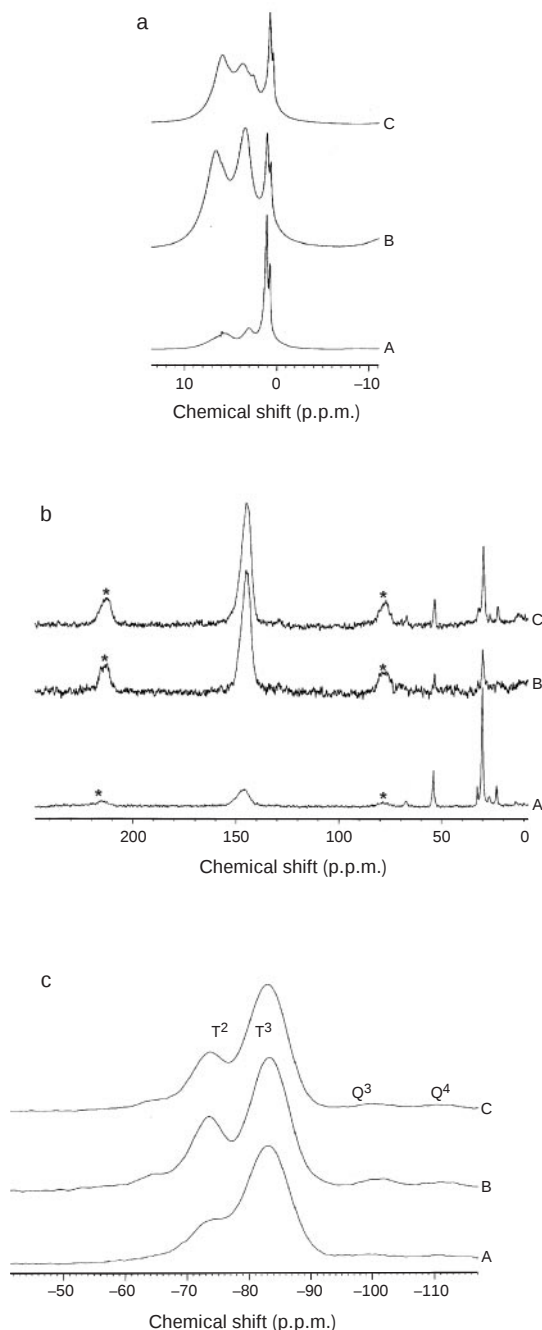


Figure 4 Solid-state NMR spectra of mesoporous ethenesilica, BTE100. (See Figs 10, 11 in Supplementary Information.) **a**, ^1H MAS NMR spectra (3 s recycle delay, 10 scans): trace A, as-synthesized; B, solvent-extracted; and C, ion-exchanged. **b**, ^{13}C CP-MAS NMR spectra (5 ms contact time; 3 s recycle delay; 1,600 scans): trace A, as-synthesized; B, solvent-extracted; and C, ion-exchanged. Both the ^1H and ^{13}C spectra show the disappearance of alkyl groups as the surfactant is extracted leaving the ethylene groups in the materials. Some residual surfactant was also present in the spectrum. **c**, ^{29}Si CP-MAS NMR spectra (10 ms contact time; 4 s recycle delay; 20,000 scans): trace A, as-synthesized; B, solvent-extracted; and C, ion-exchanged. The ^1H (400.1 MHz), ^{13}C CP-MAS (100.6 MHz) and ^{29}Si CP-MAS (79.5 MHz) spectra were obtained with a Bruker DSX400 spectrometer. Samples were spun at a rate of ~ 6.8 kHz, except for trace A in **a**, which was spun at 6 kHz.

BTE100 were now observed at -58 and -66 p.p.m., respectively. Importantly, no Q_3 or Q_4 environments were observed by ^{29}Si MAS NMR, indicating that Si–C bonds were not cleaved during the reaction. FT-Raman also supported this result, showing elimination of the C=C stretching mode at $1,580\text{ cm}^{-1}$ and appearance of new peaks assigned to C–C and C–Br stretching modes. Powder X-ray diffraction and TEM images of the samples showed that the structural integrity and order of the material is well maintained. On reaction, the hexagonal unit cell of the mesoporous material expanded by 8%, which suggests that these materials may have unusual mechanical properties. This expansion is consistent with the transformation from sp^2 to sp^3 hybridization at the framework C atoms, and the concomitant lengthening of the C–C bond. Adsorption analysis showed a decrease in the BET surface area, pore volume and diameter from $637\text{ m}^2\text{ g}^{-1}$, $0.60\text{ cm}^3\text{ g}^{-1}$, $39.4\text{ \AA}$ to $520\text{ m}^2\text{ g}^{-1}$, $0.46\text{ cm}^3\text{ g}^{-1}$, $35.6\text{ \AA}$. Chemical analysis of the sample indicated that it contained less bromine than expected for 100% bromination. It appears that while some ethene groups ($\sim 10\%$) were brominated, others reacted with the solvent to form ethane bridges. Nonetheless, these results show that ethene groups incorporated into the channel walls are accessible for chemistry.

We now compare these periodic mesoporous organosilicas, containing organic groups inside the walls of mesoporous silica, with mesoporous silica containing tethered vinyl groups inside the channels²¹. While both offer the possibility of functionalization and coordinating species, such as catalysts, there are inherent differences between the two classes. The terminal vinyl groups occupy space inside the channels, which may interfere with adsorption of species. There is also a limit of $\sim 25\%$ loading of terminal vinyl groups in the mesoporous silica before structural order is lost. Also, the reactivity of a bridging ethene that is housed within the silica framework, and attached to two electron-withdrawing O_3Si framework moieties, is expected to be different from that of a terminal vinyl located in the channel space and attached to one O_3Si group. Perhaps the most important difference is that chemical reaction of the organic materials in our materials will result in changes to the properties of the framework. If this process can be extended to conducting or semiconducting materials, the creation of a new generation of chemical sensors, membranes and chemical delivery systems may be facilitated. □

Methods

General synthesis

A typical synthesis procedure required a mole ratio of $1.00\text{ Si} : 114\text{ H}_2\text{O} : 8.0\text{ NH}_4\text{OH}$ (35 wt%): 0.12 CTABr . BTE and TEOS were used as the silica source. BTE (Gelest) was used without further purification. Samples were prepared with BTE:TEOS in Si mole ratios of 1:0 (BTE100), 0.75:0.25 (BTE75), 0.50:0.50 (BTE50), 0.25:0.75 (BTE25) and 0:1 (BTE0). CTABr (0.21 g) was added to a solution of NH_4OH (4.43 g, 30 wt%) and deionized water (8.35 g), and the solution was stirred for 30 min in a closed polyethylene bottle. A mixture of BTE:TEOS corresponding to the mole fraction given above, with a total of 4.8 mmol Si (for example, 0.635 g (1.8 mmol) BTE + 0.250 g (1.2 mmol) TEOS for sample BTE75), was slowly added to the base/surfactant solution with slow stirring. After stirring for 30 min, the solutions were aged at 80°C for 4 d in closed polyethylene bottles. The white powder was collected by filtration, washed thoroughly with water, and air-dried under ambient conditions. A typical yield was 0.690 g for BTE75. BET surface area measurements were obtained for the solvent-extracted samples at McMaster University Powder Processing Facility.

Surfactant removal

Solvent extraction of surfactant was carried out by the procedure of Stein *et al.*²¹ An as-synthesized sample (0.5 g) was stirred for 48 h in a refluxing solution of 10 g HCl (36 wt%) and 70 g of methanol. For ion exchange of the surfactant, the sample was stirred in a refluxing solution of 1 g NaCl and 100 g of ethanol.

Bromination

A solvent-extracted sample (0.50 g) was kept under vacuum at 150°C for 2 d, and then treated in refluxing $\text{Br}_2/\text{CH}_2\text{Cl}_2$ solution (7.0 g Br_2 + 150 ml CH_2Cl_2) for 8 d. The product was isolated on a Buchner funnel and washed with dichloromethane. Bromine analysis confirmed that Br was incorporated into the mesostructure, though less than the amount expected for an ideal formula of $(\text{CHBr})\text{SiO}_{1.5-x}(\text{OH})_x$. As we have shown that all of the

ethene groups were consumed, some may have reacted with the solvent. We are investigating the mechanism of the bromination reaction.

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Supplementary information is available on Nature's World-Wide Web site (<http://www.nature.com>) or as paper copy from the London editorial office of Nature.

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Seismic anisotropy of the Earth's inner core resulting from flow induced by Maxwell stresses

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Seismological observations indicate that the inner core of the Earth is elastically anisotropic¹. Anisotropic structures are likely to be formed by dynamic processes and therefore such observations have the potential to provide constraints on flow in the inner core and on the geodynamo itself. But in addition to the difficulties in estimating the relevant physical properties of iron under inner-core conditions^{2–4}, even the macroscopic processes responsible for generating seismic anisotropy in this region have yet to be determined^{5–9}. As a result, the geodynamic significance of seismic anisotropy in the inner core has remained unknown. Here I propose—based on geodynamic and mineral physics considerations—that flow induced by the stress due to the magnetic field, the Maxwell stress, near the inner-core boundary produces an axisymmetric fabric responsible for the observed seismic anisotropy. The resultant seismic anisotropy reflects the geometry of the magnetic field near the inner-core boundary and therefore seismological observations might provide constraints on the geodynamo. This flow also causes non-uniform release of energy at the inner-core boundary, associated with solidification and melting which may affect the pattern of convection in the outer core.

The seismic anisotropy of the Earth's inner core¹ and the rotation of the inner core with respect to the mantle (“super-rotation”) ^{10,11} suggest that this central portion of our planet is dynamically active. The super-rotation and the observed nearly axisymmetric seismic anisotropy suggest an important interaction between the inner and the outer core. Super-rotation is considered to be due to the interaction between the inner and the outer core through the Maxwell stress^{12,13}, but there has been no consensus as to the likely macroscopic processes that could cause seismic anisotropy. Here I will review some of the previous models of anisotropy in the inner core, and then show that the Maxwell stress could also play a critical role in the formation of the anisotropic fabric in the inner core that causes seismic anisotropy.

As theoretical analysis indicates an effective draining of possible melt from the inner core¹⁴, the most likely cause of seismic anisotropy in the inner core is the lattice preferred orientation (LPO) of iron. In identifying likely macroscopic processes of anisotropic structure formation by LPO, it is important to appreciate that large-strain deformation and/or the long-term action of stress are needed for the development of significant LPO. A plausible macroscopic process must also explain the nearly axial symmetry of anisotropy.

Some previous studies suggested that deformation-induced fabric (that is, LPO) of iron crystals due to thermal convection is the origin of seismic anisotropy^{5,15}, in much the same way as in the upper mantle. However, theoretical analysis of the energy budget and heat transfer strongly indicates that thermal convection is unlikely in the inner core, because of a low concentration of heat-generating elements and the efficient thermal conduction^{16,17}. Furthermore, the axial symmetry of anisotropy is not readily explained by this model.

An alternative model is the texturing of iron crystals during solidification from the liquid outer core^{6,7}. Although this model can explain the axial symmetry of anisotropy, a major problem of this

model is that such a texture is likely to be modified by large-strain plastic flow in the central portions of the inner core, if the inner core has a low viscosity as suggested by mineral physics¹⁸ and geodynamic considerations¹⁹.

Gravitational interaction of the inner core with the mantle may cause plastic strain which may be responsible for seismic anisotropy in the inner core⁸. However, this is unlikely to be a dominant mechanism for seismic anisotropy for two reasons. First, Buffett¹⁹ showed that the stress due to this interaction must be relaxed quickly (~ 1 yr), and that the average stress due to this interaction is $\sim 10^3$ Pa or less (corresponding to a torque of $\sim 10^{19}$ N m or less), so that the net effects of this force will not be large. Second, even if the magnitude of this stress was comparable to or larger than stresses due to other processes, the magnitude of the net strain is not large ($\sim 10^{-4}$). Furthermore, the strain in each cycle of rotation of the inner core with respect to the mantle is not cumulative, so the total contribution of this force to the production of the LPO will not be very large.

Yoshida *et al.*⁹ proposed that the stress due to anisotropic growth of the inner core causes fabric through stress-induced recrystallization. The stress due to this process is proportional to the viscosity as $\sim \eta u/a$ (here η is the viscosity of the inner core, u is the rate of inner-core growth; and a is the radius of the inner core). Using the viscosity estimated from mineral physics and geodynamic considerations^{18,19} ($10^{15} < \eta(\text{Pa s}) < 10^{17}$), the stress associated with this process would be $\sigma \approx 10^{-2}$ – 1 Pa; under such a stress, the alignment of minerals to form anisotropic structure would take an exceedingly long time, greater than the age of the Earth.

Another important source for stress that could last throughout most of the Earth's history is the Maxwell stress²⁰ caused by the magnetic field $\mathbf{B}$, $\sigma_{ij} = [B_i B_j - (1/2)\delta_{ij} B^2]/\mu_0$ (here μ_0 is the magnetic permeability of the vacuum, δ_{ij} is Kronecker's delta), or

equivalently the Lorentz force $\mathbf{f} = (1/\mu_0)\text{curl } \mathbf{B} \times \mathbf{B}$. The importance of the magnetic field in the exertion of forces on the inner core has been recognized in relation to the super-rotation which may be caused by the balance of magnetic torque, $\Gamma = (\int B_r B_\phi r \sin \theta dS)/\mu_0$, with the torque due to viscous force^{12,13}, where S is surface area and r is the distance from the centre. In this case, the product of radial field (poloidal field, B_r) and zonal (toroidal) field (B_ϕ) gives rise to a torque causing rotation. When B_ϕ is stronger than B_r , as suggested by some dynamo models^{21–25}, the normal stress due to the zonal (toroidal) field $\sigma_{rr} = -B_\phi^2/2\mu_0$ would be significantly larger than the stress causing super-rotation, and could play an important role in the deformation of the inner core. For a range of estimates¹³ of B_ϕ (10^{-2} – 10^{-1} T), the stress magnitude is estimated to be $\sigma \approx 10^2$ – 10^4 Pa. Notice that although this stress could fluctuate, the flow caused by this stress will assume a nearly constant pattern—at least over an average period of constant magnetic polarity (0.1–10 Myr)²³, which is much longer than the timescale of fluctuation of gravitational force due to inner-core/mantle interaction ($\sim 10^2$ – 10^3 yr) (refs 17, 18). As a result, the magnitude of strain associated with this process will be significantly larger than that due to deformation by gravitational interaction.

To investigate the nature of flow due to this stress, I consider a case where the zonal (toroidal) field dominates at the boundary of the inner and outer core. In this case, the Lorentz force squeezes inner-core materials towards the rotation axis of the inner core, and moves materials from the regions of high stress to the regions of low stress (Fig. 1). The materials that reach the low-stress regions will melt, and the materials that have been removed from the high-stress regions will be replaced by the solidification of molten iron in the nearby regions. The heat released or absorbed by these processes is likely to be carried away quickly through the outer core relative to the rate of flow in the inner core, because the outer-core materials have a much lower viscosity than the inner-core materials. In this case, the shape of the inner core is defined by the melting temperature of iron, and will remain unchanged despite large flow.

I have calculated the flow field by solving the Stokes equation with appropriate boundary conditions. The boundary conditions for this problem are (1) no singularities at the centre of the inner core, (2) no shear traction at the inner–outer core boundary ($r = a$, where a is the radius of the inner core) and (3) the continuation of normal traction (σ_{rr}) at the inner–outer core boundary. I assume that the normal stress at the inner–outer core boundary is determined by the Maxwell stress, $\sigma_M(\theta, \phi)$, thus, $\sigma_{rr}(a, \theta, \phi) = -B^2/2\mu_0 \equiv -\sigma_M(\theta, \phi)$ where the zonal (toroidal) field B_ϕ is written as B for simplicity, θ is co-latitude and ϕ is longitude.

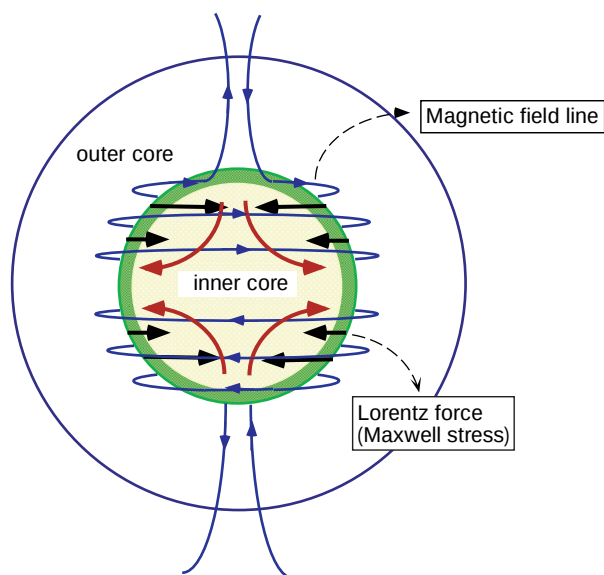


Figure 1 A schematic diagram showing the structure and dynamics of the inner core. The dynamics of the outer portion of the inner core (dark-green, thin layer) is dominated by the direct effects of the Lorentz force (the Maxwell stress). The dynamics in the deep portions are controlled by the force balance between the pressure gradient and the viscous force, with a constraint imposed by the boundary conditions near the surface of the inner core which are dominated by the Maxwell stress. The Lorentz force (the Maxwell stress) caused by the toroidal magnetic field squeezes inner-core materials towards the rotation axis, and causes a flow from strong-field regions to weak-field regions. The flow pattern is sensitive to the geometry of the magnetic field (see Fig. 2). Black arrows, Lorentz force; red arrows, flow directions; blue lines with arrows, magnetic field lines.

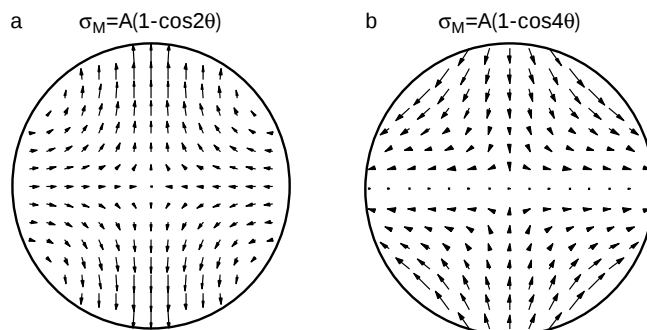


Figure 2 Flow field caused by the magnetic field at the boundary between the inner and the outer core. Flow velocity vectors in a meridional plane are shown. The geometry of the flow field is determined by the geometry of the magnetic field at the inner–outer core boundary. The flow field corresponding to the Maxwell stress of (panel **a**) $\sigma_M(\theta, \phi) = A(1 - \cos 2\theta)$ (corresponding to the symmetric toroidal field) and (panel **b**) $\sigma_M(\theta, \phi) = A(1 - \cos 4\theta)$ (corresponding to the antisymmetric toroidal field) are shown.

To illustrate the basic physics, I consider simple cases where the Maxwell stress has the following form, $\sigma_M(\theta, \phi) = A(1 - \cos 2\theta)$ or $\sigma_M(\theta, \phi) = A(1 - \cos 4\theta)$, corresponding to the symmetric ($B \propto \sin \theta$) or antisymmetric ($B \propto \sin 2\theta$) toroidal field, respectively. The flow patterns corresponding to these patterns of Maxwell stress are shown in Fig. 2. The symmetric field causes a flow pattern dominated by the degree 2 pattern: flow from equatorial regions to the polar regions. The antisymmetric field causes a flow pattern that contains both degree 2 and 4 components, but because the field is strong near the polar regions, the flow pattern is dominated by the flow from the polar regions to the equatorial regions. The stress magnitude is $\sim A \approx 10^3$ Pa. The velocity of this flow is of the order of $\sim aA/10\eta \approx 10^{-9} - 10^{-7} \text{ m s}^{-1}$. Thus the timescale of the flow is $\sim 10\eta/A \approx 1-100$ Myr. This timescale is comparable to (or somewhat greater than) the timescale of magnetic field polarity. When a reversal occurs, there will be a short period (10^3-10^4 yr; ref. 23) in which the magnetic field assumes very different geometry, but the geometry of the Maxwell stress will be similar to that for the reversed magnetic polarity. Therefore the flow pattern will not be significantly affected by a field reversal.

This Maxwell-stress-induced flow has two important consequences. First, the flow results in a large strain and hence could cause significant fabric (LPO) of inner-core materials. The geometry of anisotropy is closely related to the geometry of the magnetic field near the inner–outer core boundary. The LPO due to this process has axial symmetry, regardless of the actual mechanisms causing it. The seismic anisotropy caused by the flow depends on the microscopic mechanism of LPO and the elastic constants of iron. For the flow pattern caused by the antisymmetric toroidal field which corresponds to the $\alpha\omega$ -dynamo²⁴ or the $\alpha^2\omega$ -dynamo²⁵, for example, the elastic constants determined by Singh *et al.*³ and the dominant prismatic slip along the *c*-axis are consistent with the seismological observation of the fast P-wave velocity along the rotation axis. However, neither the elastic constants nor the dominant slip systems in inner-core materials are well constrained at present.

Second, the flow causes non-uniform energy release at the inner–outer core boundary due to the melting or solidification of iron. Because the flow velocity in this model ($\sim 10^{-9}-10^{-7} \text{ m s}^{-1}$) is comparable to (or faster than) the likely growth velocity of the inner core ($\sim 10^{-10} \text{ m s}^{-1}$; ref. 17), the amount of latent heat release due to this flow can be very large. This heterogeneous heat release at the inner–outer core boundary is likely to have an important effect on the convection pattern in the outer core: hence the inner core may have an important influence on the geodynamo through thermal effects, as well as through the effects of geometrical control of the convection pattern²⁴. For example, the flow corresponding to the antisymmetric magnetic field causes excess heat generation (or absorption) at the inner–outer core boundary that has 2θ and 4θ terms, and hence two peaks: one major peak (heat flow maximum) at polar regions and a minor peak at the equatorial region. Such a heat flow pattern is consistent with some dynamo calculations²⁵.

The present model suggests a strong link between the dynamics of the inner and outer core through mechanical coupling via the Maxwell stress. The Maxwell-stress-induced inner-core dynamics provide an explanation for both seismic anisotropy and super-rotation of the inner core^{12,13}, and suggests possible effects on geodynamo processes. However, several problems remain. First, mineral physics data such as the phase diagram²⁶, elastic constants^{2,3} and the mechanisms of deformation and recrystallization in iron in the inner core⁴ are still poorly constrained. Further studies on these mineral physics issues are required to explore the geodynamic implications of seismic anisotropy. Second, it is also important to investigate the effects of large energy release at the inner–outer core boundary on the convection, and hence on the geodynamo processes in the outer core. Thermal boundary conditions at the inner–outer core boundary, as well as at the core–mantle boundary²⁷, may

be important in the geodynamo, and hence the core may not be a slave of the mantle. In this regard, further studies of the structure and dynamics of the boundary layer between the inner core and outer core are critical to our understanding of the mechanisms of feedback between the flow in the inner core and convection in the outer core. Strong feedback may modify the flow pattern in the outermost regions of the inner core that corresponds to a given magnetic field. Such a study may provide an explanation for the sharp transition from an isotropic (shallow) layer to an anisotropic (deep) layer found by Song and Helmberger²⁸. Third, there are considerable uncertainties in the magnitude of the magnetic field in the boundary between the inner and the outer cores^{23,24}. A significantly smaller magnetic field than assumed here would result in much weaker flow in the inner core. If this was the case, other processes—such as the flow caused by gravitational force¹⁹—may make important additional contributions to anisotropy, but the details of these processes have not been investigated. □

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Ultraviolet colour variation influences blue tit sex ratios

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Brilliant blue and violet structural colours are common plumage ornaments in birds, but their signalling functions are poorly understood¹. This may be because birds also communicate in ultraviolet (UV-A) wavelengths (320–400 nm)^{2–5}, invisible to humans, but a strong spectral component of many structural colours⁶. From a wild population of blue tits—*Parus caeruleus*, sexually dimorphic primarily in the ultraviolet^{7,8}—we report experimental evidence that females skew the sex ratio of their offspring in response to the ultraviolet plumage ornamentation of their mates. Masking male ultraviolet reflectance reversed a positive correlation between reflectance and brood sex ratio observed in control pairs, demonstrating a causal effect of male ultraviolet ornamentation on offspring sex ratio. Ultraviolet reflectance also predicted male survival to the following breeding season, suggesting that it serves as a viability indicator. When taken together with ecological effects (laying date, nesting area), our experiments reveal that an unexpected amount of control exists over the primary sex ratio in birds, suggesting that chromosomal sex determination may not constrain the sex ratios of multiparous vertebrates.

When the relative fitness returns from sons and daughters vary with some ecological or morphological variable, sex allocation theory predicts that parents will distribute resources between male and female offspring in response to that variable^{9,10}. Male sexual attractiveness might be such a cue, because (provided that sexual selection acts more strongly in males) females mated to attractive males will gain in fitness by investing more in sons. But support for sex ratio adjustment in relation to paternal attractiveness is very limited^{11,12}, and only one experimental study (of a captive population, with artificial ornaments¹¹) has provided positive evidence. A previous study of blue tits found that females mated to males with higher subsequent survival produced more sons in their broods¹³, but identified no phenotypic advertisement of these viability differences among males. Recently, however, blue tits have been found to exhibit sexual dimorphism in the ultraviolet (UV), together with indications of mating preferences with respect to this colour variation^{7,8}. As sexual selection in the wild acts via several mechanisms to increase the variance in male reproductive success relative to females^{14,15}, the UV sex dimorphism in blue tits provides an ideal system for a field experiment to test whether females adjust the sex ratios of their offspring in response to their mates' attractiveness.

We studied a population of blue tits breeding in nest-boxes on the Swedish island of Gotland in 1998 and 1999. In 1998, males of breeding pairs were captured and sequentially assigned to control or experimental treatments. After measures of spectral reflectance had been taken⁷, males in the experimentally UV-reduced (UVR) group had a mixture of avian preen gland fat and UV-absorbing sunblock chemicals smeared onto the crown (an area showing pronounced sex differences in UV coloration^{7,8}). Males in the control group were

treated identically, except that the fat contained no sunblock chemicals. The UVR treatment was effective in reducing the UV component of the reflectance (Fig. 1a), whereas the control treatment did not affect the spectral shape of the reflectance function (that is, its 'colour'¹⁶). Owing to gloss from the fat coating, both treatments produced a slight uniform increase in total reflectance ('brightness'). The UVR treatment reduced the UV component of reflectance far below the level of the dullest male or female in the natural population (Fig. 1a; mean change in PC1, 6.85 standard deviations, s.d.; sexual dimorphism in PC1, 1.25 s.d. Here PC1 is the first principal component from a principal components analysis: see Methods). Furthermore, because the treatment reduced the variance in UV components of reflectance among males, naturally UV-bright males were subject to a greater reduction in UV components of reflectance than were less UV-bright males (Fig. 1b; regression: $F_{1,19} = 18.49$, $P < 0.001$). The treatment thus acts to mask variation in the UV region, rather than making males appear as dull normal males.

After controlling for the influence of other variables correlated with the sex ratio (Methods), we found, within the control group ($n = 20$ males), significant positive relationships between the proportion of sons in a male's brood and objective measures of UV crown coloration ('chroma' and 'hue', defined in Methods: Fig. 2a–d: UV chroma: likelihood ratio $\chi^2_1 = 4.04$, $P = 0.044$; peak wavelength ('hue'): $\chi^2_1 = 5.94$, $P = 0.015$; PC1: $\chi^2_1 = 5.83$, $P = 0.016$). There was no relationship between sex ratio and total reflectance ('brightness', defined in Methods: Fig. 2b: $\chi^2_1 = 0.57$, $P = 0.45$). In the UV-reduced group ($n = 21$ males) these relationships (using pre-manipulation values) were reversed (Fig. 2a–d: UV chroma: $\chi^2_1 = 4.28$, $P = 0.039$; peak wavelength: $\chi^2_1 = 8.77$, $P = 0.003$; PC1: $\chi^2_1 = 7.11$, $P = 0.008$); again, there was no relationship between sex ratio and total reflectance ($\chi^2_1 = 2.93$, $P = 0.09$). In each case the relationship between UV colouration

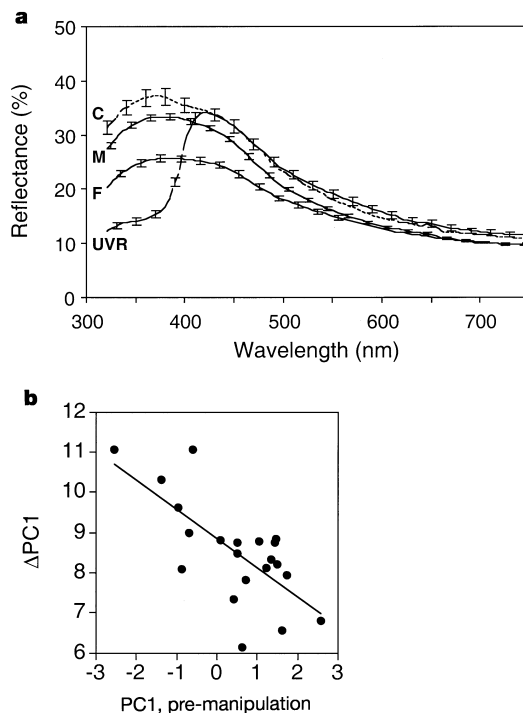


Figure 1 Natural variation and experimental effects on reflectance of blue tit crown plumage. **a**, Average spectral reflectance (320–750 nm) from blue tit crown plumage (percentage reflected radiance compared to that of a white standard). M, males ($n = 79$), F, females ($n = 19$), UVR, UV-reduced males ($n = 21$), and C, control treated males ($n = 9$). Where spectra converge, standard error bars are excluded from the female (F) and control male (C) spectra. **b**, Relationship between pre-manipulation colour (PC1) and the change in PC1 (Δ PC1) caused by the experimental treatment for 21 males in the UVR group. Note that males with UV-bright plumage have negative values of PC1.

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and sex ratio differs between the two experimental groups (Table 1). Similar patterns are found if the analysis is restricted to the smaller sample of broods for which we obtained the primary sex ratio (that is, the sex of each egg laid: treatment $\times$ UV chroma: $\chi^2_1 = 4.60$, $P = 0.03$; treatment $\times$ peak wavelength: $\chi^2_1 = 7.92$, $P = 0.005$; treatment $\times$ PC1: $\chi^2_1 = 6.47$, $P = 0.01$). Hence, the bias in sex ratio is present at ovulation.

Lack of detailed knowledge concerning blue tit colour vision hampers speculation about the 'true' perceptual effects of the UVR treatment. Although it was unexpected that UV masking reversed (rather than removed) the relationship between pre-manipulation coloration and sex ratio, this may have occurred because the treatment's effect was strongly dependent on a male's pre-manipulation characteristics (for example, Fig. 1b): naturally UV-bright males changed more than did naturally dull males. Correlations between UV reflectance of different plumage patches in males (for example, crown versus wing coverts UV chroma: $r = 0.29$, $n = 78$, $P < 0.01$) would thus result in greater contrast between the UV-deprived crown and surrounding plumage in males with naturally more intense UV coloration.

Previous studies of sexual selection in blue tits have found that females preferred males that survived to the subsequent breeding season as extra-pair copulation partners¹⁴, and that they produced an excess of sons in their broods when breeding with such males¹³. We investigated whether UV signals served as an indicator of male survival probability by recapturing all breeding blue tits in the year after we conducted our experiments. Males that had survived the winter had significantly greater UV chroma than males that were not recaptured (Fig. 3a; logistic regression: $\chi^2_1 = 4.231$, $P = 0.040$). No other measure of UV-signal exaggeration was significantly related to recapture probability (peak wavelength: $\chi^2_1 = 0.86$, $P = 0.36$; PC1: $\chi^2_1 = 2.66$, $P = 0.10$). When males that received the UVR treatment were excluded (because the treatment had interfered with the natural relationship between sex ratio and UV

signals), overwinter survival was also positively associated with the sex ratio of the brood in the previous year (Fig. 3b; logistic regression: $\chi^2_1 = 4.571$, $P = 0.033$). If any effect on survival is mediated through territory quality, this should affect females equally and cause a correlation in survival probability between pair members. There was no corresponding relationship between female overwinter survival and brood sex ratio ($\chi^2_1 = 2.35$, $P = 0.13$) or between the likelihood of survival of the two members of a pair ($\chi^2_1 = 0.21$, $P = 0.65$).

These results suggest that the UV colour signal itself serves as an indicator of male phenotypic quality in blue tits, and hence corroborates other evidence suggesting this to be a target of female choice^{7,8}. In socially monogamous birds, increased success of attractive males in obtaining extra-pair copulations is an important source of sexual selection on males¹⁷. Provided that males inherit attractiveness from their fathers (through either genetic or environmental mechanisms) this provides an important source of selection for biased sex allocation^{11,12}. All studies of blue tit populations to date have revealed extra-pair paternity to be frequent enough (29–42% of broods)^{14,18} to cause substantial opportunity for increased sexual selection on males. Further work is required to establish whether, and by which mechanisms, male blue tits inherit UV coloration from their fathers.

Since Darwin's first treatment of the topic there has been great interest in the role of sexual selection in the evolution of brilliant colour patterns and sexual dichromatism in birds¹⁹. Relatively few studies, invariably of carotenoid pigmentation (for example, refs 20, 21), have demonstrated selection on colour variation *per se* (for example, hue, chroma) unconfounded by spatial properties such as patch presence or size (though see ref. 3). In this respect, we note that our results have identified sexually selected variation in a structural colour. With the recent development of tools for objective quantification of animal colours^{16,22}, other examples of colour communication are likely to be revealed in the near future. Future studies also need to determine the mechanism by which structural colour variation might serve as an indicator of phenotypic quality^{23,24}.

The chromosomal sex of avian eggs is thought to be determined only hours before ovulation²⁵. As our experimental manipulations were carried out only shortly before laying (mean interval between manipulation and the first egg of the clutch was 10 ± 5.2 s.d. days), the sex ratio adjustment must have occurred in the period shortly before ovulation and must have been in response to intrinsic properties of the male himself. Our results add to the small number of studies^{26–28} providing experimental evidence that female birds can control the primary sex ratio of their offspring, but reveal an unusually large degree of control, judging by the variance in sex ratio explained (Table 1). The mechanism by which

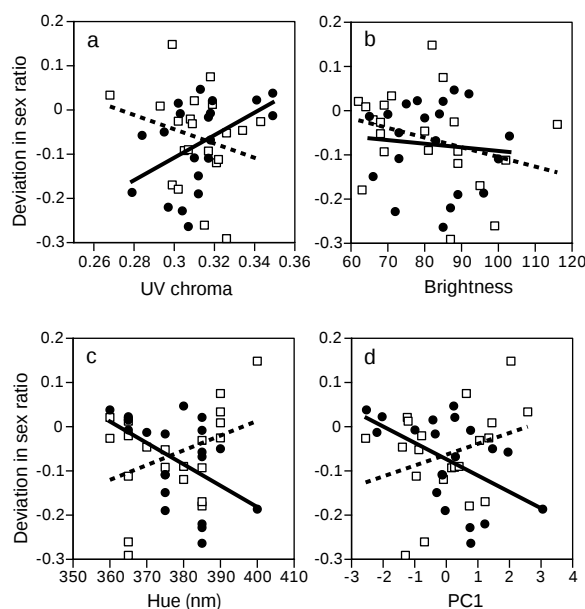


Figure 2 Relationships between spectral reflectance parameters and deviation from expected sex ratio (proportion males). Data are shown for broods of blue tits from UV-reduced (dashed lines, open symbols) and control (continuous lines, filled symbols) males. **a**, UV chroma (reflectance ratio, $R_{320-400}/R_{320-700}$); **b**, brightness (total spectral reflectance between 320–700 nm); **c**, hue (wavelength of peak reflectance, $\lambda(R_{max})$); **d**, PC1: first principal component from principal components analysis involving the three variables in **a–c**. Positive deviations in the sex ratio indicate an excess of males, calculated as the observed sex ratio minus that predicted from a model containing laying date, male and female age, and the woodland that the birds bred in (see Methods). Thus a deviation of +0.2 indicates a sex ratio 20% higher than expected.

Table 1 Analyses of sex ratios in broods of blue tits

	Colour variable in model			
	d.f.	'UV chroma'	'Hue'	PC1
Correlated variables				
Laying date	1	11.32*	17.03*	13.64*
Woodland	9	22.86†	26.90†	23.98†
Male age	1	1.73	3.36§	2.30
Female age	1	5.06‡	6.60‡	5.97‡
Experimental variables				
Treatment	1	6.33‡	10.71†	0.18
Colour measure	1	0.40	0.86	0.48
Treatment x colour measure	1	6.11‡	10.85†	7.85†
Full model	15	35.91†	39.67*	37.61*
% variance in sex ratio explained		51.1	55.2	53.2

Values are $-2\log LR$ from ordinal logistic regression models containing all independent variables in the Table. A separate model was constructed for each of the three colour variables. d.f., degrees of freedom; 'UV chroma', 'hue' and PC1 are defined in Methods.

* $P < 0.001$, † $P < 0.01$, ‡ $P < 0.05$, § $P < 0.10$.

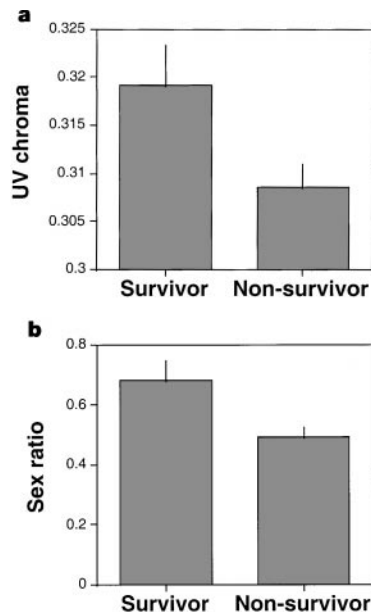


Figure 3 Associations between survival of male blue tits and UV colouration and sex ratio. Relationship (mean + s.e.) between overwinter survival status of male blue tits and **a**, crown UV chroma (reflectance ratio, $R_{320-400}/R_{320-700}$) and **b**, offspring sex ratio (proportion males).

this is achieved remains unknown, but the fact that there is an experimental effect on the UV/sex-ratio relationship, in addition to correlational effects of a range of other variables (Table 1), suggests that the mechanism does not involve selective abortion or reabsorption of eggs after ovulation. This is because such abortion or reabsorption would induce frequent laying gaps and delay breeding time in species laying large clutches²⁹, as in this blue tit population (mean clutch size 10.94 ± 1.68 s.d.; laying gaps are very infrequent). Establishing the mechanism by which sex ratios are adjusted remains a major challenge, but our results demonstrate that chromosomal sex determination does not always act as a strong constraint in avian sex allocation.

Methods

Experimental treatment and reflectance data

Male blue tits were captured in nets using song playback and a stuffed decoy in front of occupied nest boxes (occupancy defined as initiated nest building). Males were assigned sequentially to either the control or the UVR group. Reflected radiation at wavelengths of 320–750 nm (± 2 nm resolution) from the UV/blue crown was measured with a PS1000 diode-array spectrometer (Ocean Optics), a DH2000 deuterium–halogen light source, and in relation to a WS-2 white standard ($>98\%$ reflectance at 300–800 nm). Five perpendicular scans ($0^\circ/0^\circ$ illumination and recording, removing the probe between each scan) from a 4-mm-wide measuring area were taken, converted to 5-nm steps, and averaged. Objective reflectance parameters generally relevant to an opponency-based perceptual colour space were computed (by S.A. and J.Ö., blind to the sex ratio results) in accordance with ref. 7: total reflectance ($R_{320-700}$) ('brightness'), reflectance ratio ($R_{320-400}/R_{320-700}$: 'UV chroma'), and wavelength of peak reflectance ($\lambda(R_{\max})$: 'hue'). As reflectance variations in these three parameters are correlated, we also calculated the first principal component (PC1) from a principal components analysis using the three variables, to provide a single measure describing colour variation. PC1 had strongly positive loading from hue and strong negative loading from UV chroma, but was weakly influenced by brightness: a positive value of PC1 thus indicates a male with low UV chroma and long wavelength at peak reflectance. The experimental (UVR) or control 'paints' were applied with a cotton swab to the crown. The UVR paint consisted of 'CDC' (fly dressing based on duck preen gland fat) (Hoh Inc.) mixed with two UVA-absorbing sunblock chemicals (Parsol 1789 and MCX, Givaudan-Roure), whereas the control 'paint' contained the CDC alone. The average crown reflectances immediately after the UVR and control treatments are shown in Fig. 1, and a more detailed account of the manipulation effects and duration is given elsewhere⁵. Males in the two groups did not differ in terms of subsequent laying date, clutch size, any pre-manipulation measures of reflectance, age or any measured morphological traits (t -tests, $P \geq 0.09$). After release of the males, blue tit breeding attempts were monitored at regular intervals to determine clutch size and laying date, and adults were recaptured during chick feeding (14-d-old young) to ensure that

males were correctly matched with their broods. A total of 80 males were manipulated (40 controls, 40 UVR); of these, sex ratios were obtained for 47 (23 controls, 24 UVR), but all data required for model-fitting (Table 1) for 41 (20 controls, 21 UVR). Breeding attempts of the remaining males failed, largely owing to nest-box takeover by great tits *Parus major* and collared flycatchers *Ficedula albicollis*, and predation of clutches.

Sex identification

Blood samples ($2-15 \mu\text{l}$) were collected from nestlings (1–2 days old) and the adults caught feeding these nestlings. Sex was determined by polymerase chain reaction (PCR) amplification of two homologous genes (*CHD1W* and *CHD1Z*). The latter occurs in both sexes whereas the former occurs on the W chromosome carried by females, the heterogametic sex in birds. Following chelex extraction of DNA from blood samples, PCR primers P2 and P8 (ref. 30) were used to amplify introns of variable length in the two genes from conserved exonic regions. Products were resolved on 6% denaturing polyacrylamide gels. Either one band (*CHD1Z*; males) or two distinct bands (*CHD1Z* and *CHD1W*; females) were seen after silver staining. Parents were sexed alongside their nestlings, when available, and in all cases ($n = 63$, males, $n = 67$ females for all broods analysed), molecular sexing agreed with sex determined at capture on the basis of presence or absence of a brood patch. Nestlings (16) sexed from blood samples in 1998 were recaptured as breeding adults in 1999; in all cases (11 males, 5 females), the sex determined using genetic markers agreed with that determined from the presence/absence of a brood patch. We obtained the primary sex ratio (that is, we knew the sex of all eggs laid) for 26 (63%) of the 41 broods for which we analysed relationships between sex ratio and colour. In the remaining 15 broods, the primary sex ratio was unavailable either because unhatched eggs could not be sexed or nestlings that died soon after hatching and were removed by the parents could not be sampled (mean proportion of clutch sexed for these nests, 0.79 ± 0.03 s.e., range: 0.55–0.92). All nestlings that were blood sampled were successfully sexed. Weighting each brood in the analysis by the proportion of the clutch sexed does not change the statistical significance and interpretation of the results.

Analysis

Sex ratio variation was analysed using ordinal logistic regression with the proportion of males in the brood as the dependent variable. In a larger data set ($n = 590$ nestlings in 57 broods), for which all relevant variables were measured, there were significant, or marginally non-significant, independent effects on brood sex ratio of clutch initiation date ($\chi^2_1 = 13.00$, $P = 0.0003$), the woodland in which the birds bred ($\chi^2_2 = 20.68$, $P = 0.01$, male age (adult or yearling: $\chi^2_1 = 3.04$, $P = 0.08$) and female age ($\chi^2_1 = 3.93$, $P = 0.05$). These variables were therefore entered first in all models analysing the relationship between colour variables and the sex ratio, and constrained to remain in the model. The results are robust to the exclusion of the three nests in the UVR group which appear from Fig. 2c to have high leverage; model residuals did not differ from a normal distribution (Shapiro–Wilks test, $W \geq 0.97$, $P \geq 0.19$), nor did the variance in sex ratio differ between the two experimental groups (Levene's test: $F_{1,39} = 0.04$, $P = 0.84$).

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Perception of three-dimensional shape influences colour perception through mutual illumination

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Objects in the natural world possess different visual attributes, including shape, colour, surface texture and motion. Previous perceptual studies have assumed that the brain analyses the colour of a surface independently of its three-dimensional shape and viewing geometry^{1,2}, although there are neural connections between colour and two-dimensional form processing early in the visual pathway^{3,4}. Here we show that colour perception is strongly influenced by three-dimensional shape perception in a novel, chromatic version of the Mach Card—a concave folded card with one side made of magenta paper and the other of white paper. The light reflected from the magenta paper casts a pinkish glow on the white side. The perceived colour of the white side changes from pale pink to deep magenta when the perceived shape of the card flips from concave to convex. The effect demonstrates that the human visual system incorporates knowledge of mutual illumination—the physics of light reflection between surfaces—at an early stage in colour perception.

To quantify this phenomenon, we constructed a concave card with trapezoidal sides which appeared rectangular when viewed from a distance (Fig. 1a). We painted the left side of the card magenta, the right white. Mutual illumination between the two sides generated a strong chromatic gradient across the white side, so that its measured chromaticity varied from deep pink near the crease to pale pink at its outer edge (Fig. 1b). By diluting the magenta paint with increasing amounts of white paint, we created a set of 23 alternative matching colours on small chips, densely

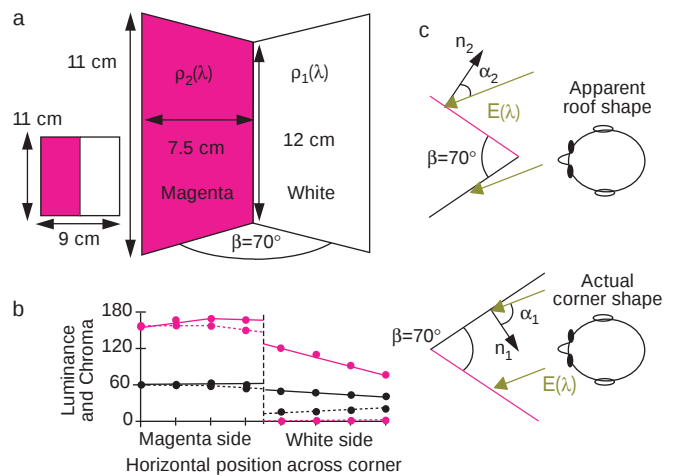


Figure 1 The experimental stimulus. **a**, The chromatic Mach card. The large drawing gives its actual dimensions, with exaggerated perspective cues; the small drawing shows its actual appearance. Average luminance and chromaticity values: 56.58, 0.49, 0.52 (magenta side) and 42.43, 0.45, 0.52 (white side) (CIE 1976 $L^*u^*v^*$ coordinates). **b**, Solid lines, measured variation in chroma (magenta line) and lightness (black line) horizontally and centrally across the card (see Methods). Dashed lines, measurements without mutual illumination (obtained by replacing alternate sides with black paper). **c**, Plan views of the stimulus, illustrating the inversion of the card under pseudoscopic viewing, and the approximate direction of the light source E .

sampling a rough line from magenta to white in uniform colour space (inset, Fig. 2). The card was attached to the back wall of a black box (the stimulus box) and illuminated by a hidden incandescent lamp regulated by a dimmer. A panel containing the coloured chips was attached to the back of a second black box (the matching box) and illuminated by two hidden incandescent bulbs. The illumination in each box was adjusted to produce the same chromaticity and luminance from a central white card, which was removed prior to the experiment.

In the 'roof' condition, observers viewed the card through a pseudoscope, a binocular viewing stand fitted with Dove prisms. The prisms invert the image in each eye from left to right, thereby reversing binocular stereo disparities and with them, the depths of objects in the image. The card therefore appeared convex. In the 'corner' condition, observers viewed the card through empty but otherwise identical viewing tubes. The card now appeared concave, its true shape. In the roof condition, the white side of the card appeared on the left; in the corner condition, the white side appeared on the right (Fig. 1c). Because the contour cues indicated a flat card and there were no visible shadows in the black stimulus box, the primary cue to the card's three-dimensional shape was from binocular disparities. Observers were instructed to match the colour of the left (roof) or right (corner) side by selecting from the matching panel a chip identical in colour appearance (see Methods). In the control condition, observers followed the same procedure to match the colour of an unfolded, pink card, viewed with and without the pseudoscope.

The results (shown for 23 observers in Fig. 2) demonstrate a significant shift in perceived colour of the white side from a desaturated pink in the corner condition to a more saturated magenta in the roof condition. There is no shift in perceived colour of the flat control card under the two viewing conditions (Fig. 3a; the two distributions are statistically indistinguishable [$F(1, 90) = 0.05$ at $P = 0.8$]).

The effect cannot be explained in the same way as the classical Mach Card effect. The original Mach Card is a convex folded grey card, one side brightly illuminated and the other in shadow⁵. When the card is perceived correctly to be convex (roof), the two sides

appear the same grey; when perceived to be an inward-pointing corner, the shaded side appears to be painted a darker grey than the lit side. Mach concluded that the human visual system assumes that the direction of illumination is the same for both configurations, so that in the roof, the dark side is turned away from the light source, but not in the corner. In a quantitative demonstration of the phenomenon, Beck⁶ confirmed that observers' assumptions about the number and positions of light sources illuminating the card did indeed influence their lightness matches to both sides. Other studies support the explanation that implicit knowledge about light source and scene geometries or perceived surface layout may directly influence surface lightness perception^{7–11,23}.

The chromatic effect we report here cannot be explained by the above lightness effects, for two reasons. Firstly, in our setup, the light source is deliberately hidden, so that observers cannot be certain of any fixed direction of illumination. In fact, under these conditions, the classical Mach Card effect does not obtain, as we have shown in another experiment¹². Secondly, even if observers were to deduce the approximate location of the light source, they would conclude that the white side received more direct illumination in the corner shape than in the roof shape, because of the inversion induced by pseudoscopic viewing (Fig. 1c). Mach's explanation would therefore predict that the white side should appear darker in the corner than in the roof. This prediction cannot account for our results, because the less saturated matching chips neither are nor appear darker, as we demonstrated in a separate ranking experiment under the same illumination conditions.

Instead, the chromatic effect appears to depend on an inbuilt, perceptual understanding of the laws of mutual illumination. Mutual illumination—the indirect illumination created by light reflected from surfaces onto each other, as opposed to direct illumination from the primary light source—has largely been neglected in analyses of surface colour and shape perception, with a few exceptions^{13,14}. Recent analyses have shown that the chromatic component of mutual illumination may provide cues to the intrinsic surface reflectance of participating surfaces, and therefore enhance colour constancy¹⁵. Psychophysical studies of colour constancy in real^{16,17} and computer-simulated scenes¹⁸ suggest that the human visual system may indeed exploit the information that mutual illumination conveys to recover constant surface colours.

Our present study demonstrates that surface colour perception is contingent on three-dimensional shape perception. A simple model based on bayesian inference¹⁹ demonstrates that this shape–colour

contingency arises because the human visual system must intrinsically understand the effects of mutual illumination.

The model computes how an ideal observer would perform when asked to select the paper most likely to constitute the 'white' side under the two conditions. The ideal observer is limited only by the internal matching noise and is provided with the following physical information for the task: (1) an understanding of the physics of light reflection, and specifically of mutual illumination; (2) an *a priori* assumption of a single light source and independent knowledge of its energy spectrum; (3) independent knowledge of the card's shape; and (4) independent knowledge of the surface reflectance function of the magenta side, as well as of the matching chips (see Methods). This information determines the likelihood that the surface reflectance of a particular chip would give rise to the observed colour of the 'white' side; this likelihood determines the probability that the ideal observer selects that chip.

The predicted colour of the 'white' side for a particular surface reflectance varies with the direction of the light source. For the model we assume that each direction is equally probable, and integrate over all light source directions to obtain the *a posteriori* probability for each matching chip. This calculation is equivalent to the "generic view" method²⁰, using the direction of the incident illumination as the generic variable. The only difference between the calculation of the predicted match for the corner and roof configurations is that mutual illumination is allowed in the former but not in the latter.

This single feature—the presence or absence of mutual illumination—is necessary and sufficient to make the predicted probability distributions significantly different for the roof and corner configurations (Fig. 3b). Furthermore, the behaviour of the ideal observer predicts the crucial features of the observed matches: the difference

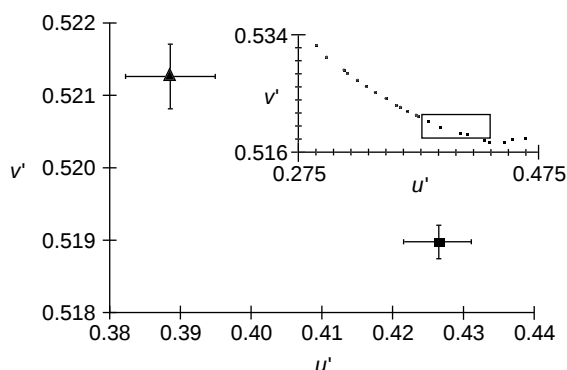


Figure 2 Average (u' , v') coordinates of the matches selected for the white side of the card. Data is shown when seen as a corner, without pseudoscope (triangles), and as a roof, with pseudoscope (squares). Error bars show s.e.m. ($n = 24$ corner, $n = 20$ roof). The difference between the roof and corner average matches is approximately ten times larger than threshold. Inset, the distribution of u' and v' chromaticity coordinates (CIE 1976 $L^*u^*v^*$) of the 23 matching chips under the illumination conditions used during the experiment. The rectangle indicates the area represented in the main graph.

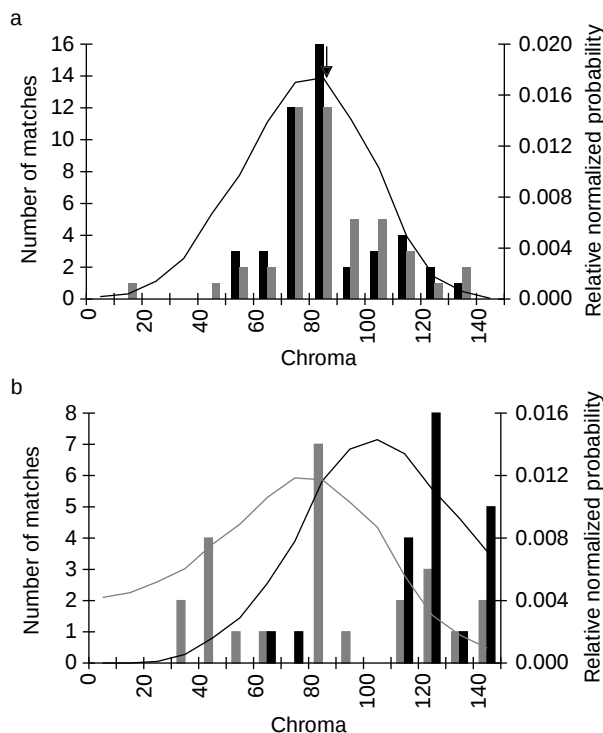


Figure 3 Observed matches and model predictions. **a**, Distribution in chroma of matches to flat control card with (black bars) and without (grey bars) pseudoscope, for 46 total matches by 23 observers, for each condition. Bin size 10; the magenta chip (number 23) has chroma 149. Arrow indicates actual chroma (86) of control card. **b**, Distribution in chroma of matches to the white side of the chromatic Mach card. Grey bars, corner shape, without pseudoscope ($n = 24$). Black bars, roof shape, with pseudoscope ($n = 20$). Overlaid curves show the predicted probability distributions as described in Methods.

between their means (31 chroma units, statistically indistinguishable from the observed mean difference of 35 ± 17), and the spread and overlap of the non-normal distributions (standard deviations of 26 versus observed 22 ± 10 , and 32 versus observed 35 ± 7 , for the roof and corner conditions, respectively; see Methods).

The model predictions demonstrate that the real observer behaves close to the ideal, and, in particular, that the real observer's visual system incorporates accurate knowledge of the effects of mutual illumination. The systematic shift in the absolute values of the observed means compared with the predicted means (Fig. 3b) suggests that the real observer lacks the ideal observer's access to accurate and complete physical measurements, in particular the illuminant spectrum.

It is important to emphasize that the real observer's task was to match the colour of the 'white' side, a direct sensory match, not to select the paper from which it was most likely to be constructed, an indirect match requiring a judgement of surface identity¹. The observer perceives two different colours from the same retinal stimulus under the two different configurations. The model performs a high-level, indirect match, and yet predicts the observed difference in low-level, direct matches. This fact suggests that there is a top-down influence of surface recognition on colour perception, similar to the reported effects of lightness judgements on perceived brightness in the achromatic domain¹¹. This influence is mediated by an in-built knowledge of the chromatic effects of mutual illumination, which dictates that the perception of surface colour and three-dimensional shape are fundamentally linked. □

Methods

Matching procedure

After securing a fused image of a test circle against a grid through the pseudoscope, the observer viewed the folded test card or flat control card in the stimulus box for at least 10 seconds, at a viewing distance of 57 cm. The observer reported whether the folded card was a 'roof' or 'corner' shape. He then moved to the adjacent matching box where the matching chips (each 3 degrees square) were displayed in a 5×5 array with 1-degree-wide gaps, against a black background. Each of four such panels, each with a different random arrangement of chips, was displayed in one of four different rotational positions, randomised across trials, to yield a total of 16 different matching displays. The observer selected the chip (by marking its equivalent position on a paper grid) that best matched the specified left or right side of the card. For each change in stimulus, the experimenter drew a black cloth across the front of the stimulus box, so that the observer's view of the card was always through the viewing tubes. Observers were randomly assigned to one of two groups: corner or roof condition first, with the control conditions interleaved between two trials of the same condition. Matches are reported for the first-viewing-only conditions.

Calculation of lightness and chroma

The lightness (L^*) of a surface is its relative brightness compared to a white surface under the same illumination. Chroma (C) is defined as the colourfulness of a surface judged as a proportion of the brightness of a similarly illuminated surface that appears white. Their numerical correlates are calculated as:

$$L^* = 116 \left(\frac{Y}{Y_n} \right)^{\frac{1}{3}} - 16$$

$$C = \sqrt{(u^*)^2 + (v^*)^2}$$

$$u^* = 13L^*(u' - u'_n)$$

$$v^* = 13L^*(v' - v'_n)$$

where Y_n , u'_n and v'_n are the chromaticity coordinates of a flat white reference paper ($C = 0$ and $L^* = 100$) under the illumination conditions of the stimulus box, in CIE $L^*u^*v^*$ colour space²¹.

Ideal observer model

For the corner configuration, a vertically infinite surface of reflectance $\rho_2(\lambda)$ forms an angle β of 70 degrees with another of reflectance $\rho_1(\lambda)$. The illuminant $E(\lambda)$ forms angles α_2 and α_1 with the two surface normals, respectively (see Fig. 1). The one-bounce model of mutual illumination¹⁵ yields the intensity equation for surface 1: $I_1(\lambda, x) = E(\lambda)\rho_1(\lambda) [\cos \alpha_1 + f_{21}(x)\rho_2(\lambda) \cos \alpha_2]$, where the first term represents the direct illumination and the second term represents indirect (mutual) illumination due to light reflected from surface 2. $f_{21}(x)$ is the β -dependent form factor describing the extent to which surface 2 reflects light onto surface 1 at distance x from the vertex. $\rho_2(\lambda)$ is specified as the measured reflectance function of chip 23 (identical to the magenta side of the card). $E(\lambda)$ is a

tungsten illuminant spectrum reconstructed from the measured chromaticity values of a white reference paper under the illumination conditions in the stimulus box, using the Cohen basis functions²¹. The surface reflectance $\rho_i^s(\lambda)$ is the measured surface reflectance of the i th matching chip ($i = 1, 2, \dots, 23$). Reflectance functions and the illuminant spectrum are specified in 10-nm steps. For the corner shape, surface 1 receives direct illumination for values of α_1 between 0 (normal to surface 1) and 90 degrees, and indirect illumination for α_1 from 20 to 110 degrees (normal to surface 2). For each value of α_1 between 0 and 110 degrees, in 1-degree steps, and for each surface reflectance $\rho_i^s(\lambda)$, the predicted light intensity $I_1^p(\lambda, x)$, at each of four positions x on surface 1, is computed according to the above formula. From $I_1^p(\lambda, x)$, the predicted luminance, hue and chroma of the 'white' side is computed using the CIE 1964 supplementary standard colorimetric observer and standard colorimetric formulae—the predicted colour of the 'white' side if its surface reflectance were $\rho_i^s(\lambda)$ and it participated in mutual illumination with the magenta side. The differences between the predicted chroma $C(\alpha_1, x)$ and the measured chroma $C_{\text{obs}}(x)$ at each position x on the 'white' side are computed and converted to an *a posteriori* probability P for the i th matching chip, by summing over all (110) illuminant directions α_1 and all four sample positions x , and normalizing areas under the curves to unity: $P(\rho_i^s | C_{\text{obs}}) = (1/k) \sum_x \sum_{\alpha_1} \exp - (|C_{\text{obs}} - C(\alpha_1, x)|^2 / (2\sigma^2))$, where k is the normalizing constant. Here σ is the only free parameter, representing the internal observation error; it is therefore chosen as the value that best predicts the distribution of chroma matches to the flat control card (that is, $\sigma = 20.36$).

For the roof configuration, the intensity equation for surface 1 is $I_1(\lambda) = E(\lambda)\rho_1(\lambda) \cos \alpha_1$. Because surface 1 receives neither direct nor indirect illumination for values of α_1 between 90 and 110 degrees, in this computation α_1 is allowed to vary only from 0 to 90 degrees. Otherwise, the calculation is the same as for the corner configuration.

Calculation of observed mean and standard deviation errors

The 95% confidence intervals for the standard deviations and differences between roof and corner means were estimated by resampling²².

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Dominant negative mutations in human PPAR γ associated with severe insulin resistance, diabetes mellitus and hypertension

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Thiazolidinediones are a new class of antidiabetic agent that improve insulin sensitivity and reduce plasma glucose and blood pressure in subjects with type 2 diabetes¹. Although these agents can bind and activate an orphan nuclear receptor, peroxisome proliferator-activated receptor gamma (PPAR γ), there is no direct evidence to conclusively implicate this receptor in the regulation of mammalian glucose homeostasis². Here we report two different heterozygous mutations in the ligand-binding domain of PPAR γ in three subjects with severe insulin resistance. In the PPAR γ crystal structure, the mutations destabilize helix 12 which mediates transactivation. Consistent with this, both receptor mutants are markedly transcriptionally impaired and, moreover, are able to inhibit the action of coexpressed wild-type PPAR γ in a dominant negative manner. In addition to insulin resistance, all three subjects developed type 2 diabetes mellitus and hypertension at an unusually early age. Our findings represent the first germline loss-of-function mutations in PPAR γ and provide compelling genetic evidence that this receptor is important in the control of insulin sensitivity, glucose homeostasis and blood pressure in man.

Type 2 diabetes mellitus is characterized by impairments of both insulin secretion and action. Although several monogenic forms of this disorder have been described, most are due to defective pancreatic beta-cell function³ with insulin receptor gene mutations being the only established genetic disorder of insulin action in man⁴. We have established a large cohort of subjects with severe insulin resistance, defined by the coexistence of extreme hyperinsulinaemia and the skin lesion acanthosis nigricans, in which candidate genes for defective insulin action are being systematically examined^{5,6}.

PPAR γ is a key regulator of adipocyte differentiation⁷. Thiazolidinediones are specific high-affinity ligands for PPAR γ ⁸, and the order of their receptor-binding affinities *in vitro* mirrors their antihyperglycaemic activity *in vivo*⁹. We therefore proposed that loss-of-function mutations in PPAR γ might impair tissue insulin sensitivity and screened this candidate gene in our insulin-resistant cohort.

In 85 unrelated subjects with severe insulin resistance, all coding exons of PPAR γ 1 and PPAR γ 2 were amplified using polymerase chain reaction (PCR) and studied by single-stranded conformation polymorphism analysis and direct sequencing of all abnormal conformers. New missense mutations in the ligand-binding domain of PPAR γ were found in two subjects. Subject 1 (the index case from Kindred A) was heterozygous for a single nucleotide substitution (CCG to CTG) corresponding to a proline to leucine mutation at codon 467 (P467L) (Fig. 1a). Her son, aged 30 years and with a

history of early onset diabetes and hypertension (Subject 2), was also heterozygous for the P467L mutation. All other family members including both parents of Subject 1, none of whom were known to have diabetes or hypertension, were homozygous for wild-type receptor sequence (Fig. 1b). Non-paternity was excluded, indicating a *de novo* appearance of the mutation in the proband. Subject 3 (the index case from an unrelated Kindred B) was heterozygous for a single nucleotide substitution (GTG to ATG) resulting in a valine to methionine mutation at codon 290 (V290M) (Fig. 1a). Her clinically unaffected mother and sister were both wild type at this locus and screening of the deceased father was not possible (Fig. 1c). No other sequence changes in PPAR γ were found in the three subjects, and these mutations were not found in at least 230 alleles from control Caucasian subjects or 84 alleles from control subjects of four other ethnic groups.

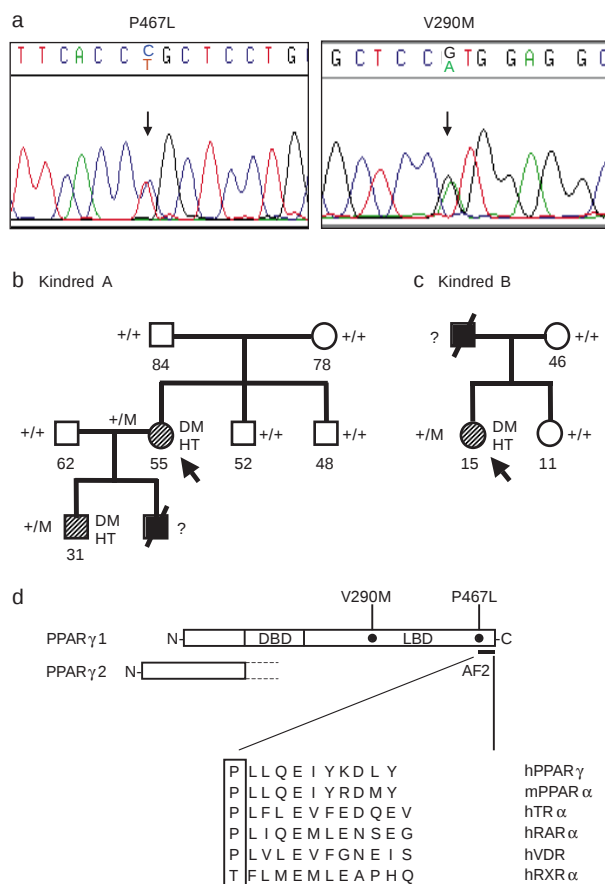


Figure 1 Two new mutations, P467L and V290M, in human PPAR γ . **a**, Heterozygous nucleotide substitutions. Left, CCG to CTG corresponding to a proline to leucine mutation at codon 467; right, GTG to ATG corresponding to a valine to methionine mutation at codon 290. **b**, **c**, Family pedigrees confirm complete concordance between the clinical phenotype and the presence of the heterozygous P467L (**b**) and V290M (**c**) receptor mutations. The age and genotype (+, wild type; M, mutant) of members is indicated. The affected individuals (striped symbols) were diabetic (DM) and hypertensive (HT), with no known diabetes or hypertension in other family members (empty symbols). Arrows indicate the probands and filled symbols denote deceased individuals. **d**, Location of the P467L and V290M mutations in PPAR γ . The γ 1 and γ 2 receptor isoforms share common DNA-binding (DBD) and ligand-binding (LBD) domains linked to divergent N-terminal regions. Val 290 is located in the centre of the LBD and Pro 467 at the origin of a C-terminal amphipathic α -helix. An alignment of nuclear receptor sequences indicates that this residue and the helical motif are highly conserved. Studies have shown that this region is important for ligand-dependent transcription activation (AF-2) and coactivator recruitment¹¹.

Subject 1, a 56-year-old female, first came to medical attention at age 20 with irregular menstrual periods and primary infertility. She underwent assisted conception five years later, developing gestational diabetes in this first pregnancy, and then frank type 2 diabetes a year postpartum. She continued to have oligomenorrhoea but conceived spontaneously at age 32, requiring insulin treatment during pregnancy which continued postpartum. Since then, it has been increasingly difficult to achieve glycaemic control despite large daily doses (currently 280 U) of insulin. In addition to diabetes, abnormal blood pressure has also been a prominent clinical feature. Severe pre-eclampsia developed in both pregnancies, necessitating early induction of labour at 37 weeks gestation in the first, and an emergency caesarean section at 30 weeks in the second, with the latter child failing to survive the neonatal period. Sustained hypertension was diagnosed at age 37, before the development of diabetic nephropathy, which requires combined therapy with three anti-hypertensive agents. Subject 2 was found to be markedly hypertensive (blood pressure 185/98) during a routine examination at 27 years of age, and requires treatment with two anti-hypertensive agents. Diabetes was diagnosed concomitantly with a fasting glucose of 8.7 mmol l⁻¹. Subject 3 presented at age 15 with primary amenorrhoea, hirsutism, acanthosis nigricans and elevated blood pressure (150/105). Her glucose tolerance was initially normal but with markedly elevated fasting and postprandial insulin levels. By age 17, she had developed type 2 diabetes and her hypertension required treatment with beta-blockers.

The clinical and biochemical features of all three subjects are shown in Table 1. All individuals have marked insulin resistance as assessed by homeostatic model assessment of fasting glucose and insulin¹⁰. As subjects 1 and 2 were studied long after frank diabetes mellitus had developed, beta-cell decompensation had probably also contributed to their current metabolic state. Of note, fasting triglycerides are raised in all three, with low high-density lipoprotein (HDL) cholesterol levels in two of the subjects, a dyslipidaemic pattern characteristic of insulin resistance. Given the proposed role of PPAR γ in adipogenesis, it is noteworthy that all subjects have a normal body mass index with no evidence of lipotrophy or abnormal fat distribution.

The P467L receptor mutation involves a residue located in an amphipathic α -helix at the carboxy terminus of the PPAR γ ligand-binding domain. This sequence motif is highly conserved in nuclear receptors (Fig. 1d) and is critical for mediating ligand-dependent transactivation (AF-2) and coactivator recruitment in a number of them¹¹. In contrast to its wild-type counterpart, a chimaeric fusion protein consisting of the P467L ligand-binding domain coupled to a heterologous Gal4 DNA-binding domain exhibited minimal

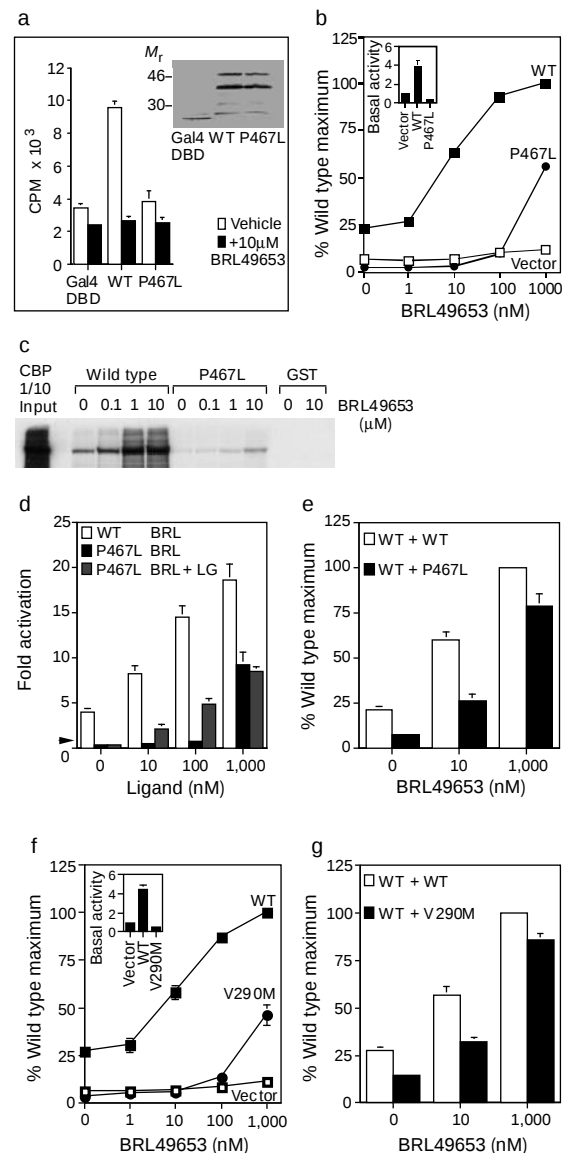


Figure 2 Functional properties of the P467L and V290M mutant receptors. **a**, Binding of thiazolidinedione radioligand [¹²⁵I]-SB236636 to Gal4-PPAR γ ligand-binding domain (LBD) chimaeras. *In vitro* translated Gal4 DNA-binding domain or wild-type (WT) and mutant (P467L) Gal4-PPAR γ LBD fusion proteins were incubated with [¹²⁵I]SB236636 in the absence or presence of 10 μ M BRL49653. In contrast to the WT receptor, the P467L mutant fusion exhibited significantly reduced ligand binding despite comparable protein expression. Inset, ³⁵S-labelled *in vitro* translated fusion proteins. *M_r*, relative molecular mass. **b**, Saturating concentrations of BRL49653 are required for transcriptional activation by the P467L mutant. 293EBNA cells were transfected with WT, P467L mutant or empty expression vectors together with a reporter gene (PPAR γ 3TKLUC). Increasing concentrations of BRL49653 were added, and results are expressed as a percentage of the maximum activation obtained with WT receptor. Inset, basal reporter activity in the absence of ligand. **c**, The P467L mutant exhibits impaired coactivator recruitment. WT and mutant GST-PPAR γ LBD fusion proteins were tested for interaction with ³⁵S-labelled *in vitro* translated CREB-binding protein (CBP) in the presence of increasing concentrations of BRL49653. Control assays were performed with GST alone. **d**, The RXR ligand LG100268 potentiates the transcriptional response of the P467L mutant to BRL49653. 293EBNA cells were transfected as in **b** and treated with ligand(s) as shown. Arrow denotes reporter gene activity in the presence of empty expression vector. **e**, The P467L mutant receptor inhibits the function of its WT counterpart in a dominant-negative manner. 293EBNA cells were transfected with 100 ng of WT plus an equal amount of either WT or P467L mutant expression vectors, together with the same reporter gene as in **b**. The transcriptional responses to 100 ng or 200 ng of WT receptor are identical (data not shown). **f**, **g**, The V290M mutant exhibits similar transcriptional impairment and dominant-negative activity. Experiments were performed as in **b** and **e**.

Table 1 Clinical and biochemical characteristics of subjects

	Subject 1	Subject 2	Subject 3	Reference range
Gender	Female	Male	Female	
Age (years)	55	31	15	
Height (m)	1.54	1.73	1.63	
Weight (kg)	59	77.5	68	
BMI (kg/m ²)	24.9	25.9	25.6	Non-obese <30
WHR	0.92	0.97	n/a	
Blood pressure (mm/Hg)	158/92*	141/79*	150/105	
Glucose	6.2	10.4	4.6	3.5–5.5 mmol/l
Insulin	325	127	703	0–60 pmol/l
% Insulin sensitivity (HOMA)	14	29	<5%	100
Cholesterol	6.0	4.1	4.4	Desirable <5.2 mmol l ⁻¹
Triglycerides	2.1	4.4	3.5	Desirable <2.0 mmol l ⁻¹
HDL cholesterol	1.5	0.7	0.8	Desirable >1.0 mmol l ⁻¹
LDL cholesterol	3.6	1.4	n/a	Desirable <4.0 mmol l ⁻¹
NEFA	318	475	n/a	280–920 μ mol l ⁻¹

All biochemical analyses were performed on fasting plasma or serum as appropriate. All subjects have type 2 diabetes mellitus with marked fasting hyperinsulinaemia, and homeostatic model assessment¹⁰ of fasting glucose and insulin confirmed that the patients have severe insulin resistance. BMI, body mass index; WHR, waist/hip ratio; NEFA, non-esterified fatty acids; n/a, not available. HDL, high-density lipoprotein; LDL, low-density lipoprotein.

* On antihypertensive treatment.

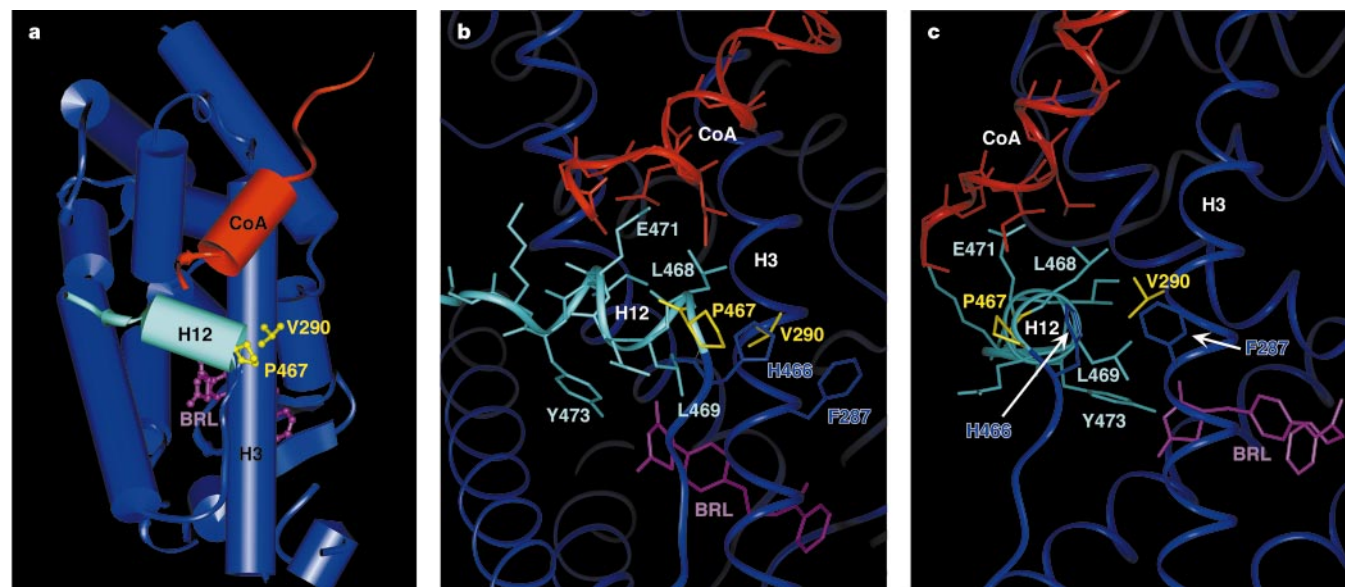


Figure 3 Pro 467 and Val 290 are important in defining the orientation of helix 12. **a**, View of the PPAR γ ligand-binding domain in complex with ligand (BRL49653I magenta) and the receptor interaction domain from the coactivator SRC-1 (CoA, red). Pro 467 and Val 290 are yellow and helix 12 is cyan. Note the locations of Pro 467 marking the N terminus of helix 12 and Val 290 close by in helix 3; mutation of either residue is likely to perturb the orientation and dynamic properties of helix 12. **b**, Detailed view of the side chains in helix 12 that interact with ligand and the coactivator helical domain. Leu 469 and

Tyr 473 interact with the thiazolidinedione head group. Pro 467 and Leu 468 form part of the hydrophobic surface on which the coactivator helix packs. Glu 471 interacts with the helix dipole of the coactivator helix. Thus, any perturbation of the orientation of helix 12 would compromise both ligand binding and coactivator interactions. Note that His 466 packs on top of Val 290. **c**, View rotated 90° compared with **b**. Note that the V290M mutation would result in the methionine clashing with both His 466 and Phe 287.

specific binding to [125 I]SB236636, a thiazolidinedione radioligand (Fig. 2a), whereas wild-type PPAR γ had a dissociation constant (K_d) of ~ 40 nM as expected. Consistent with its severely impaired ligand binding, the mutant receptor showed a markedly right-shifted activation profile when tested with a reporter gene containing a PPAR response element and increasing concentrations of the thiazolidinedione ligand BRL49653 (Fig. 2b). With $1 \mu\text{M}$ ligand, the mutant receptor attained about 50% of the maximal transcriptional activity of the wild-type receptor (Fig. 2b), indicating that saturating concentrations of ligand may partially restore receptor function. In keeping with this observation, the P467L mutant receptor recruited CREB-binding protein (CBP)¹², a known nuclear receptor coactivator, at the highest dose of ligand, but the interaction was markedly attenuated in comparison to wild-type receptor (Fig. 2c).

Unlike other nuclear receptors, PPAR γ and the retinoid X receptor (RXR) form a permissive heterodimer that can be activated by both PPAR γ and RXR-specific ligands¹³. The RXR ligand LG100268 enhances the insulin-sensitizing effects of BRL49653 when administered to obese, insulin-resistant mice¹⁴. We therefore tested whether LG100268 could enhance transcriptional activation by the mutant receptor with BRL49653. At moderate (10–100 nM) concentrations, a combination of PPAR γ and RXR ligands induced significantly greater transcriptional activation by the P467L mutant receptor than thiazolidinedione (Fig. 2d) or LG100268 (data not shown) alone. These observations raise the intriguing possibility that, when available, treatment with a thiazolidinedione in combination with an RXR ligand may be more effective than either agent alone in overcoming mutant PPAR γ dysfunction to ameliorate insulin resistance in the affected subjects.

The V290M receptor mutation involves a residue that is more proximally located within the ligand-binding domain (Fig. 1d). Functional studies confirmed that, when co-transfected with the same reporter gene, this mutant also exhibits a markedly impaired response to increasing concentrations of BRL49653, with a trans-activation profile similar to that of the P467L mutant (Fig. 2f).

With heterozygosity for the P467L and V290M mutations in

affected individuals and the dominant mode of transmission in Kindred A, we proposed that the PPAR γ mutants would inhibit wild-type receptor function in a dominant-negative manner. Consistent with this, coexpression with either the P467L or V290M mutant markedly attenuated the transcriptional function of wild-type PPAR γ (Fig. 2e, g). Our observations with the PPAR γ mutants are consonant with properties of other nuclear receptor mutants. Diverse dominant-negative thyroid hormone- β receptor mutants are associated with the syndrome of resistance to thyroid hormone¹⁵, and the oncogene v-erbA inhibits thyroid hormone and retinoic acid receptor function¹⁶. In each case, the dominant negative potency of these mutant forms of nuclear receptor is related to their ability to silence basal gene transcription^{15,16}. In this context, we find that both the P467L and V290M mutant receptors also inhibit basal reporter gene activity (inset, Fig. 2b, f).

To elucidate the structural basis for the deleterious functional effects of the P467L and V290M mutations, we examined the crystal structure of the liganded PPAR γ ligand-binding domain, bound to the receptor interaction domain of coactivator (SRC-1)¹⁷. Pro 467 marks the amino-terminal boundary of helix 12 (Fig. 3a, b), whereas Val 290 is located in helix 3 and forms part of the surface against which helix 12 is packed, making van der Waals contact with His 466, Leu 468 and Leu 469 (Fig. 3a, c). Helix 12 is important for both ligand binding and coactivator recruitment. Leu 469 contributes to a hydrophobic pocket accommodating the thiazolidinedione head group, and Tyr 473 makes a direct hydrogen bond with the nitrogen of the thiazolidinedione. On the opposite side, Pro 467 and Leu 468 contribute to a hydrophobic surface interacting with two non-polar side chains from SRC-1, whereas the negatively charged Glu 471 interacts directly with the helix dipole of the coactivator. Mutation of Pro 467 or Val 290 is likely to perturb the orientation of helix 12 and would thus compromise both functions of the receptor.

A heterozygous proline to glutamine missense mutation at codon 115 in PPAR γ 2 has been described in three unrelated subjects with obesity paradoxically associated with retention of insulin sensitivity¹⁸. This mutation disrupts mitogen-activated protein kinase

dependent phosphorylation of an adjacent serine residue¹⁹, resulting in a gain-of-function receptor mutant that mediates enhanced adipocyte differentiation *in vitro*. In population studies, a relatively common Pro12Ala PPAR γ 2 sequence variant has been variably associated with either increased²⁰ or decreased²¹ body mass index and improved insulin sensitivity. Somatic loss-of-function mutations in PPAR γ have been described in some cases of colon cancer²². Our observations represent the first examples of naturally occurring loss-of-function germline mutations in human PPAR γ . Their association with an unusual syndrome of severe insulin resistance, early onset diabetes and hypertension is striking. Of note, all subjects had a normal body mass index with no evidence of lipodystrophy, suggesting unexpectedly normal adipose tissue development. We speculate that residual PPAR γ activity, perhaps mediated by high endogenous ligand concentrations in adipocytes, is sufficient to maintain adipogenesis.

The elevated blood pressure found at an early age in all three subjects is of interest. PPAR γ is expressed in endothelial and vascular smooth muscle cells^{23,24}. Moreover, thiazolidinediones lower blood pressure in man²⁵, block calcium channel activity in smooth muscle cells²⁶, inhibit release of the vasoconstrictor endothelin-1 (ref. 27) and promote secretion of the vasodilator C-type natriuretic peptide from endothelial cells²⁸. Thus, a causal link between the PPAR γ mutation and early onset hypertension in our affected individuals through effects on vascular tone is plausible.

These families represent a new subtype of dominantly inherited type 2 diabetes, due to defective transcription factor function resulting in impaired insulin action rather than secretion. The co-segregation of deleterious dominant-negative mutations in the PPAR γ gene with extreme insulin resistance, early onset diabetes and hypertension provides compelling evidence for the direct involvement of this nuclear receptor in the control of insulin sensitivity, glucose homeostasis and blood pressure in man. These findings provide substantial support for efforts to develop specific, potent ligands for PPAR γ as agents for the treatment of human insulin resistance, type 2 diabetes, hypertension and related metabolic disorders. □

Methods

Screening of PPAR γ gene

Exons encoding both the PPAR γ 1 and γ 2 isoforms were amplified from genomic DNA (primer pairs available on request) and the PCR products subjected to single-stranded conformation polymorphism analysis. Those showing anomalous migration were sequenced directly to identify the nucleotide change. Paternity testing of subject 1 was performed by genotyping her parents using a panel of highly polymorphic combined DNA index system markers.

Ligand-binding assays

Mutant receptors were generated by site-directed mutagenesis of a wild-type receptor template using a standard protocol (Stratagene). *In vitro* translated Gal4 DNA-binding domain-PPAR γ ligand-binding domain fusion proteins were incubated with [¹²⁵I]-SB236636, a PPAR γ -specific radioligand²⁹, in binding buffer (50 mM HEPES pH 7.9, 100 mM KCl, 2 mM dithiothreitol (DTT), 10% glycerol) for 45 min at 25 °C. Competitor cold ligand (10 μ M BRL49653) or solvent carrier (dimethylsulphoxide) was added, and the reaction incubated for a further 2 h at 25 °C. Bound and free [¹²⁵I]SB236636 were separated using a modification of a filter binding assay¹⁵. Results represent the mean $\pm$ s.e.m. of three experiments performed on independently generated protein samples.

Transient transfection assays

293EBNA cells were transfected in 24-well plates with 500 ng of (PPARE) γ TKLUC³⁰ and 100 ng of receptor expression vector (wild-type, P467L mutant, V290M mutant or empty vector pCDNA3) using the calcium phosphate method¹⁵. Luciferase values were normalized to β -galactosidase activity from the internal control plasmid BosGal¹⁵, and represent the mean $\pm$ s.e.m. of at least three independent experiments, each performed in triplicate.

Coactivator recruitment assay

GST-PPAR γ ligand-binding domain fusion proteins were prepared as described¹⁹ with minor modifications. *Escherichia coli* were grown for 3 h at 37 °C before induction with 0.4 mM isopropylthio- β -D-galactosidase at 30 °C for a further 2 h. After purification, proteins bound to glutathione-Sepharose (GTS) beads in binding buffer (40 mM HEPES, pH 7.8, 100 mM KCl, 5 mM MgCl₂, 0.2 mM EDTA, 1% Nonidet P-40, 10% glycerol, 2 mM

DTT) were mixed with 5 μ l of ³⁵S-labelled *in vitro* translated CREB-binding protein together with ligand or vehicle and incubated at 4 °C for 2 h. Following washing with NETN buffer (20 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40), bound CREB-binding protein was determined by SDS-PAGE.

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EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF

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Cross-communication between different signalling systems allows the integration of the great diversity of stimuli that a cell receives under varying physiological situations. The transactivation of epidermal growth factor receptor (EGFR)-dependent signalling pathways upon stimulation of G-protein-coupled

receptors (GPCRs), which are critical for the mitogenic activity of ligands such as lysophosphatidic acid, endothelin, thrombin, bombesin and carbachol, provides evidence for such an interconnected communication network¹⁻⁴. Here we show that EGFR transactivation upon GPCR stimulation involves proHB-EGF and a metalloproteinase activity that is rapidly induced upon GPCR-ligand interaction. We show that inhibition of proHB-EGF processing blocks GPCR-induced EGFR transactivation and downstream signals. The pathophysiological significance of this mechanism is demonstrated by inhibition of constitutive EGFR activity upon treatment of PC3 prostate carcinoma cells with the metalloproteinase inhibitor batimastat. Together, our results establish a new mechanistic concept for cross-communication among different signalling systems.

Epidermal growth factor receptor transactivation was identified as a critical element in GPCR-induced mitogenic signalling^{1,5,6}, and in regulation of various ion channels^{7,8}. As the process is very rapid^{1,7,9} and GPCR-induced release of EGFR ligands into the cell culture medium cannot be detected^{5,8}, EGFR transactivation has been proposed to be exclusively mediated through intracellular signals^{3,4}. Even though platelet-derived growth factor receptors (PDGFRs) are not tyrosine phosphorylated upon treatment of Rat-1 cells with GPCR ligands², a chimera EP-R (ref. 10) consisting

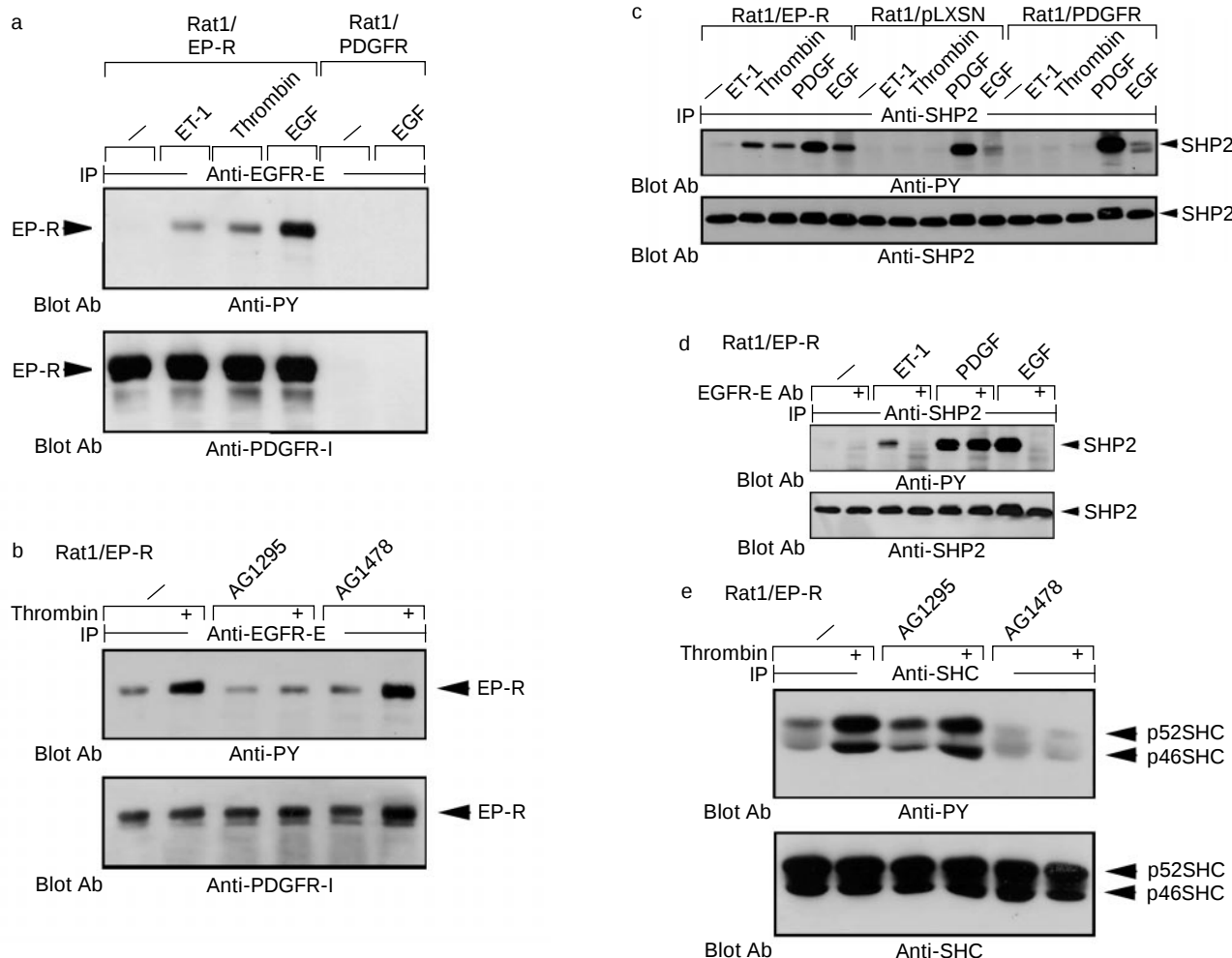


Figure 1 GPCR-induced EP-R transactivation redefines endogenous EGFR-mediated signalling to PDGFR-specific signals. Rat-1 cells stably expressing the EP-R chimeraic receptor (Rat-1/EP-R), the PDGFR (Rat-1/PDGFR) or vector alone (Rat-1/pLXSN) were treated with agonist for 3 min, and, after immunoprecipitation (IP), proteins were immunoblotted with anti-PY antibody (Ab) (4G10). Re-probing against the intracellular part of PDGFR (anti-PDGFR-I), SHP-2 or SHC, respectively, ensured precipitation of equal protein amounts. **a**, **b**, Rat-1/EP-R cells were stimulated with ET-1 (200 nM), thrombin

(2 U ml⁻¹) and EGF (2 ng ml⁻¹) (**a**), or pre-incubated with tyrphostins as indicated (**b**) before thrombin stimulation, and EP-R was selectively precipitated with monoclonal antibody 108.1. **c**, **d**, Rat-1 cell lines were untreated (**c**) or pre-incubated for 1 h with EGFR-E Ab ICR-3R (20 µg ml⁻¹), stimulated with GPCR agonists, EGF or PDGF-BB (25 ng ml⁻¹) (**d**) and SHP-2 was precipitated. **e**, Rat-1/EP-R were treated as in **b** and SHC was immunoprecipitated.

of an EGFR extracellular portion and the PDGFR transmembrane and cytoplasmic signalling domain is transactivated (Fig. 1a). Treatment of Rat-1/EP-R cells with the PDGFR inhibitor AG1295 (ref. 11), but not with the EGFR antagonist AG1478 (ref. 1), blocked thrombin-induced tyrosine phosphorylation of the chimaeric receptor (Fig. 1b). Figure 1c shows that EP-R transactivation results in a PDGF-characteristic downstream signal, as the SH2-domain-containing phosphatase 2 (SHP-2), a preferred mediator of PDGFR signalling¹², was tyrosine phosphorylated upon endothelin (ET-1) and thrombin stimulation of Rat-1/EP-R cells but not Rat-1 cells overexpressing the PDGFR. Pretreatment of Rat-1/EP-R cells with monoclonal antibody ICR-3R¹³, which blocks ligand binding to the human EGFR, resulted in the complete inhibition of ET-1- and EGF-induced SHP-2 tyrosine phosphorylation, whereas the PDGF-mediated response was not affected (Fig. 1d), confirming that GPCR-induced transactivation of the EP-R chimaera depends on the extracellular EGFR domain. In contrast, tyrosine phosphorylation of SHC (Src homologous and collagen-like) proteins after thrombin stimulation was blocked by pretreatment of Rat-1/EP-R cells with AG1478, but remained unaffected by pre-incubation with the PDGFR antagonist AG1295 (Fig. 1e). This confirms that

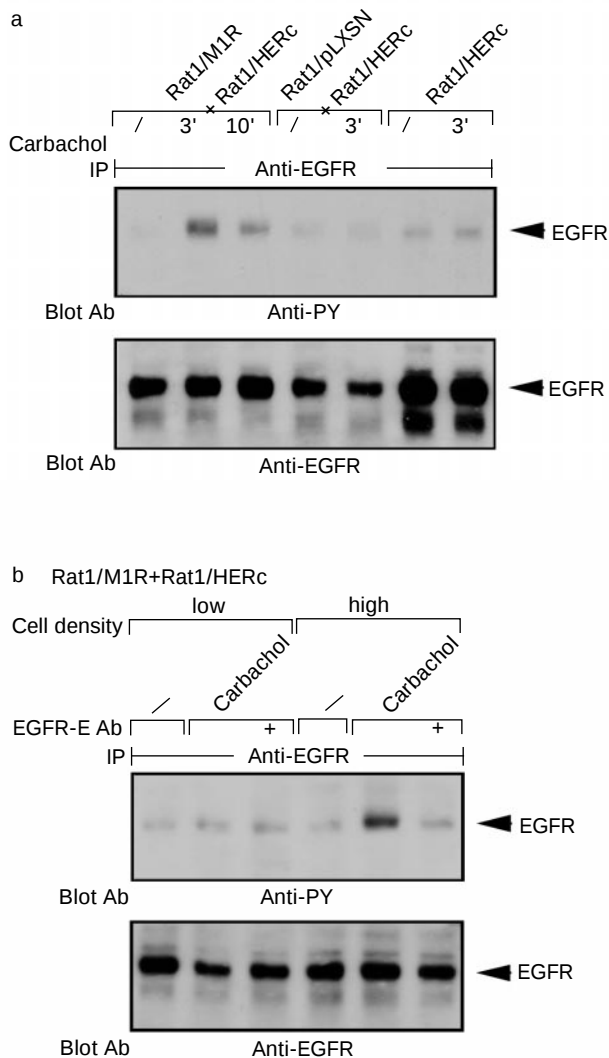


Figure 2 Carbachol-induced intercellular transactivation of the EGF receptor. Stable Rat-1 cell lines expressing either M1R or HERc and control cells were mixed in a 1:1 ratio. **a**, After stimulation with carbachol (1 mM), HERc was precipitated and immunoblotted with anti-PY antibody. **b**, Co-cultures of Rat-1/M1R and Rat-1/HERc cells were plated in different densities, pre-incubated with EGFR-E blocking antibody ICR-3R (20 μ g ml⁻¹) and HERc was precipitated after 3-min stimulation with carbachol.

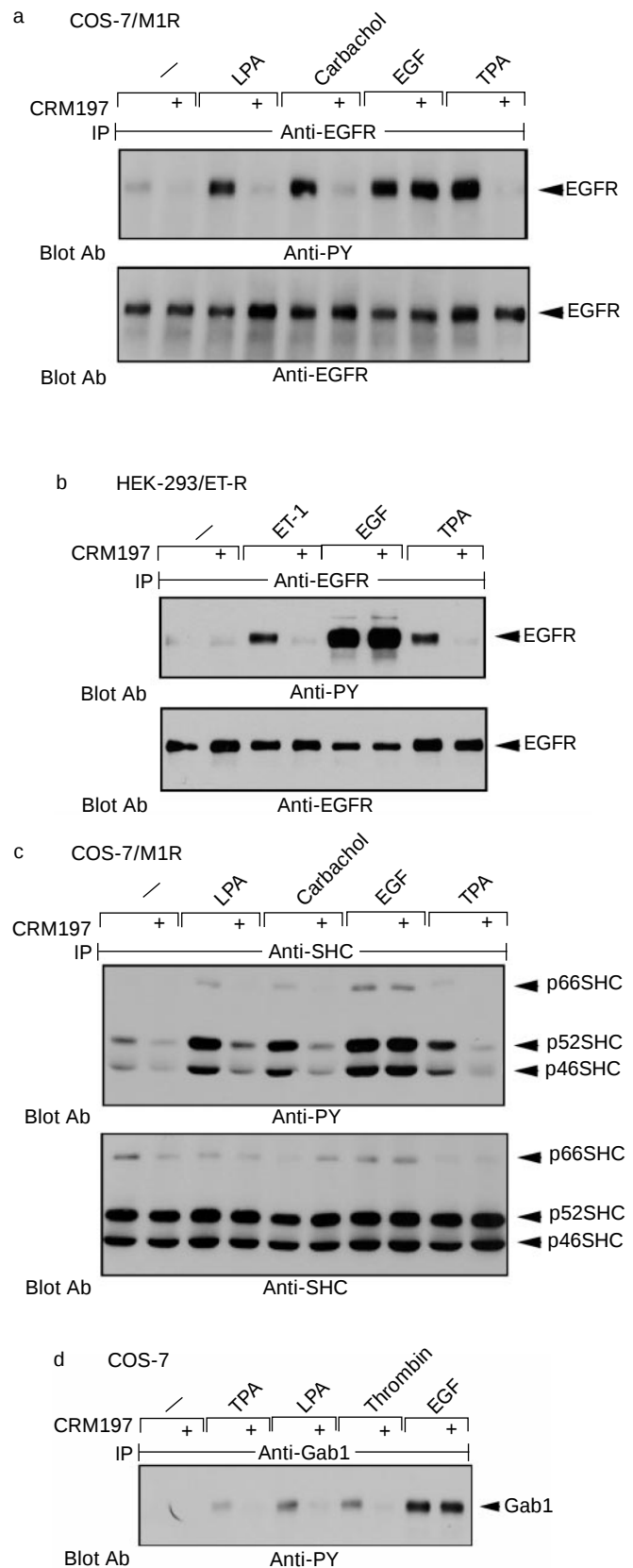


Figure 3 GPCR-induced EGFR transactivation and adapter protein tyrosine phosphorylation is dependent on HB-EGF function. COS-7 (**a**, **c**, **d**) and HEK 293 (**b**) cells, transfected with M1R and ET-R, respectively, and either CRM197 pre-incubated or untreated, were stimulated for 3 min with the GPCR agonists LPA (10 μ M) or carbachol (1 mM), EGF (2 ng ml⁻¹) or 1 μ M TPA (5 min) as indicated. Subsequently EGFR (**a**, **b**), SHC (**c**) or Gab1 (**d**) was immunoprecipitated and proteins were immunoblotted with anti-PY antibody (4G10).

thrombin transactivates endogenous rat EGFR in Rat-1/EP-R cells, which results in SHC tyrosine phosphorylation, whereas activation of the EP-R chimera redefines thrombin stimulation to generate a PDGFR-characteristic SHP-2 signal.

To address whether the extracellular signal can act in a paracrine mode, we co-cultured Rat-1 cells overexpressing either the M1 muscarinic acetylcholine receptor (M1R) or the human EGFR (HERc). Stimulation of the Rat-1/M1R+Rat-1/HERc co-culture with the M1R agonist carbachol before immunoprecipitation with human EGFR-specific antibody 108.1 rapidly induced EGFR tyrosine phosphorylation (Fig. 2a). As neither of the control cells responded to carbachol, transactivation occurs between two cells. To investigate the influence of cell density, HERc was immunoprecipitated from subconfluent and confluent co-cultures after stimulation with carbachol. Figure 2b shows that EGFR tyrosine phosphorylation in response to an M1R agonist only occurred in confluent co-cultures and was inhibited by pre-incubation with the ICR-3R antibody. This further shows the requirement of the ligand-binding function of EGFR for intercellular signal transmission and the necessity of close cell-cell contact. Thus, we conclude that EGF-like ligands, synthesized as transmembrane precursors and converted to the mature form by proteolytic cleavage¹⁴, may be involved in GPCR-mediated transactivation. The discrepancy between previous results, in which EGF-like ligands could not be detected upon GPCR activation^{5,8}, and our findings may be because upon proteolytic processing EGF-like ligands may remain associated with the heparan sulphate proteoglycan matrix before interaction with their high-affinity receptors.

Ectodomain shedding is induced by stimuli such as activators of heterotrimeric G-proteins, AIF₄ and GTPγS¹⁵, as well as tetradecanoyl-phorbol-13-acetate (TPA) and the Ca²⁺-ionophore ionomycin^{16,17}. Ionomycin, which induces HB-EGF release in prostate epithelial cells¹⁷, is a potent activator of EGFR transactivation in PC12 cells¹⁸. HB-EGF, a member of the EGF family, has the ability to bind to cell-surface heparan sulphate proteoglycans¹⁹, which prevents the immediate release of the growth factor and increases the local growth factor concentration in the cellular microenvironment. Besides its function as a growth factor precursor, proHB-EGF serves as the high-affinity receptor for diphtheria toxin²⁰. CRM197, a non-toxic mutant of diphtheria toxin, inhibits strongly and specifically the mitogenic activity of HB-EGF²¹. We found that CRM197 pretreatment completely inhibits tyrosine phosphorylation of the EGFR induced by the GPCR agonists lysophosphatidic acid (LPA) and carbachol, as well as TPA in COS-7 cells (Fig. 3a). Inhibition was also observed for ET-1- or TPA-stimulated HEK 293 cells transiently transfected with the endothelin receptor (Fig. 3b). In contrast, EGF-induced receptor tyrosine phosphorylation was unaltered, demonstrating CRM197 specificity. Furthermore, complete abrogation of LPA- and carbachol-induced receptor tyrosine phosphorylation indicated that HB-EGF may be the only growth factor mediating EGFR transactivation in these cell lines.

To investigate the role of HB-EGF in the coupling of GPCR activation to Ras-dependent signalling pathways, we examined the effect of CRM197 on GPCR ligand and TPA-mediated SHC tyrosine phosphorylation²². Figure 3c shows that, in COS-7 cells, LPA-, carbachol- and TPA-induced SHC tyrosine phosphorylation was markedly reduced by CRM197 pretreatment, whereas the EGF-mediated response was not affected. Similarly, in COS-7 cells, tyrosine phosphorylation of the multidocking protein Gab1 in response to LPA or thrombin was not detected in the presence of CRM197 (Fig. 3d), which confirms its position downstream of the EGFR².

To examine whether proHB-EGF is proteolytically processed upon stimulation of GPCRs, we transfected plasmids containing VSV-tagged proHB-EGF in COS-7 cells together with the M1R or the bombesin receptor (BombR) and stimulated them with respective ligands for different times. TPA, a potent inducer of proHB-

Figure 4 GPCR-induced proteolytic processing of proHB-EGF and EGFR transactivation are critically dependent on metalloproteinase function. **a**, COS-7 cells were co-transfected with either M1R or BombR (0.5 μg each) and VSV-proHB-EGF (0.7 μg) and stimulated with carbachol (1 mM), bombesin (200 nM), TPA (1 μM) or EGF (2 ng ml⁻¹). ProHB-EGF was analysed with anti-HB-EGF antibody (upper panel); cleaved VSV-HB-EGF was monitored by anti VSV immunoblotting (lower panel). **b**, COS-7 cells transfected as in **a** were pre-incubated with batimastat (5 μM, 30 min), stimulated as indicated, and anti-VSV immunoprecipitates were subjected to anti-HB-EGF immunoblotting. **c**, Flow cytometric analyses of proHB-EGF in COS-7 cells treated for 10 min with LPA, TPA, EGF or batimastat pre-incubation after LPA stimulation. Control cells were labelled with FITC-conjugated secondary antibody alone. **d, e**, COS-7 cells, transfected with the M1R, untreated or BB-94 pre-incubated, were stimulated as in Fig. 3a, and EGFR (**d**) or SHC (**e**) was immunoprecipitated. Proteins were immunoblotted with anti-PY antibody (4G10). **f**, PC-3 cells were serum-starved for 36 h, pre-incubated with batimastat and stimulated for 3 min with bombesin, TPA or EGF (7 ng ml⁻¹) as indicated. EGFR was immunoprecipitated and immunoblotted with anti-PY antibody. **g**, Unstarved PC-3 cells were treated for indicated times with DMSO or batimastat, and EGFR tyrosine phosphorylation was monitored with anti-PY immunoblot.

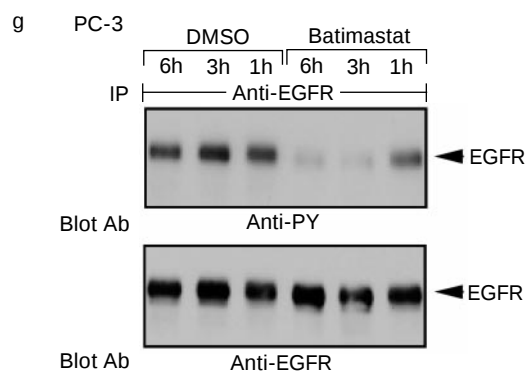
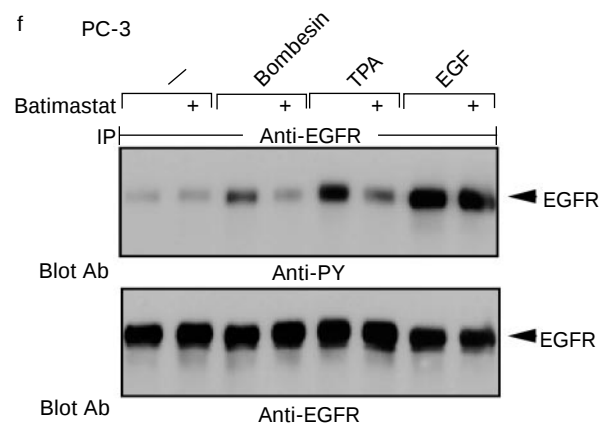
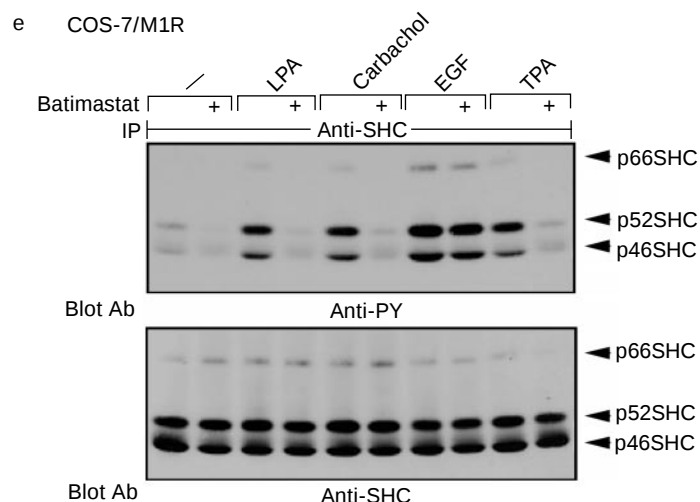
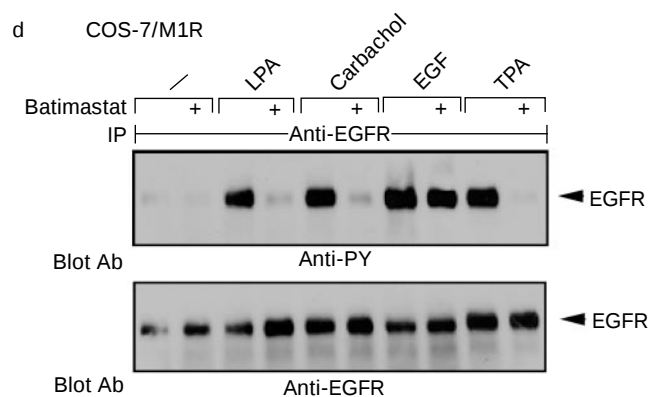
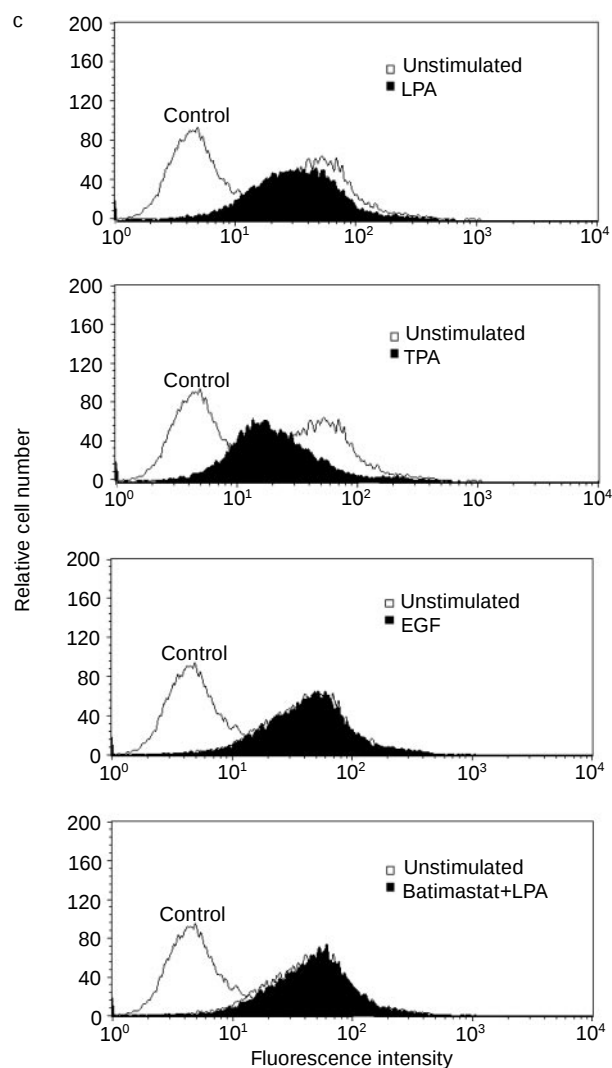
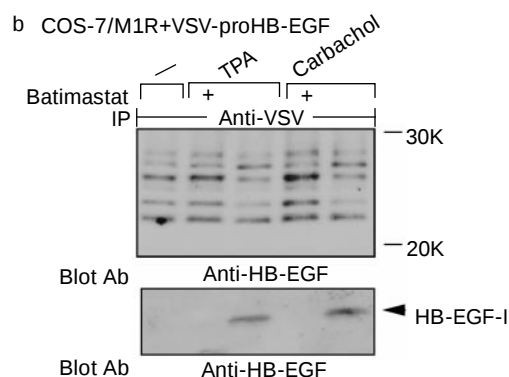
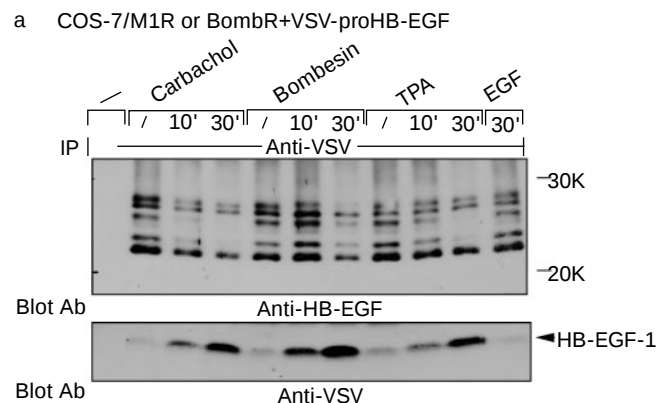
EGF processing, and EGF were added as positive and negative controls, respectively. Figure 4a shows that, as previously described, proHB-EGF is expressed in form of heterogeneous translation products of relative molecular mass 20–30K (ref. 16), which can be detected with antibodies against either the precursor or the VSV-tag. Stimulation with carbachol or bombesin led to a rapid breakdown of the growth factor precursor and proteolytic cleavage was concomitant with the appearance of the 9K VSV-tagged HB-EGF fragment containing the transmembrane anchor.

Because zinc-dependent metalloproteinases have been implicated in proHB-EGF shedding by TPA²³, we analysed carbachol-induced processing in the presence of batimastat (BB-94)²⁴, a potent inhibitor of metalloproteinases. As shown in Fig. 4b, BB-94 treatment reduced HB-EGF processing in response to carbachol, supporting our conclusion that metalloproteinases are critical elements in GPCR-induced HB-EGF generation.

To confirm that GPCRs induce proHB-EGF processing, we used an ectodomain-specific antibody and flow cytometry upon treatment of non-transfected COS-7 cells with LPA, TPA or EGF. Within 10 min after addition of LPA and TPA the content of cell-surface proHB-EGF was reduced, whereas EGF stimulation showed no effect (Fig. 4c). Consistent with the results in Fig. 4b, the modest LPA-induced effect was completely inhibited by batimastat.

Our results show that metalloproteinase-dependent cleavage of proHB-EGF is rapidly induced upon activation of GPCRs and consequently indicate a critical and general role of this process in EGFR transactivation. We therefore investigated the effect of the metalloproteinase inhibitor batimastat on GPCR- and TPA-induced EGFR transactivation. In COS-7 cells, BB-94 pretreatment completely abrogated LPA- and carbachol-induced tyrosine phosphorylation of the EGFR, as well as TPA-mediated receptor activation (Fig. 4d). As TPA- but not GPCR-mediated EGFR tyrosine phosphorylation is sensitive to protein kinase C inhibition in COS-7 cells (data not shown), it appears that at least two distinct metalloproteinase-dependent transactivation pathways exist. Finally, the general implication of proteolytic processing in EGFR transactivation and downstream signal transmission is shown by the complete abrogation of GPCR- and TPA-induced SHC tyrosine phosphorylation by batimastat (Fig. 4e).

Because of the well established role of EGFR family members in the pathogenesis of a variety of cancers and the physiological abundance of GPCR ligands such as LPA, we addressed the pathophysiological significance of transactivation with the human prostate cancer cell line PC-3, which has been reported to use EGFR-dependent pathways for growth promotion and which is also responsive to the GPCR ligand bombesin^{25,26}. Figure 4f shows that, in PC-3 cells that were starved for 36 h, bombesin and TPA induce tyrosine phosphorylation of the EGFR which is completely



blocked by batimastat-treatment. Moreover, even the high constitutive phosphotyrosine content of the EGFR in unstarved PC-3 cells is reduced by long-term treatment with batimastat (Fig. 4g). Together, our results allow the conclusion that metalloproteinase-mediated precursor cleavage represents a direct link between BombR activation, constitutive tyrosine phosphorylation of the EGFR and proliferation of human prostate cancer cells.

Batimastat-sensitive metalloproteinases have been implicated in the processing of amphiregulin and TGF α precursors²⁷, and ADAM9, a member of the metalloproteinase-disintegrin family, has been reported to process proHB-EGF upon TPA treatment of Vero-H cells²³. We were unable, however, to block EGFR transactivation with dominant-negative ADAM9 mutants in COS-7 and HEK 293 cells (data not shown), which leaves the identity of the proteolytic activity in this pathway unresolved.

Our findings identify the HB-EGF precursor and metalloproteinase activity to be critical pathway elements between GPCR signals and activation of the EGFR, and extend our understanding of the mechanisms that underly the multiple biological processes known to be regulated by heterotrimeric G-proteins. The GPCR-induced EGFR transactivation mechanism represents a new paradigm because it entails three transmembrane signal transmission events. Ligand activation of heterotrimeric G-proteins by interaction with a GPCR results in an intracellular signal that induces the extracellular activity of a transmembrane metalloproteinase; this leads to the extracellular processing of a transmembrane growth factor precursor and release of the mature factor which, directly or through the proteoglycan matrix, interacts with the ectodomain of the EGFR and activates an intracellular signal. Our previous findings indicate that this pathway may be used by a variety of GPCRs in diverse cell types and that the preferred transactivation target is the EGFR and its relatives¹⁻⁴. The pathophysiological relevance of this new mechanism in prostate cancer cells leads us to propose that EGFR transactivation through G-protein-mediated proteolytic growth factor precursor processing represents a general mechanism with broad significance. Moreover, as a great variety of bioactive polypeptides as diverse as tumour necrosis factor- α , FAS-ligand or L-selectin are processing products of transmembrane precursors²⁸ that have been connected to pathophysiological disorders, our findings shed new light on the importance of membrane-associated proteinases as targets for disease intervention strategies. $\square$

Methods

Cloning and plasmids

Plasmids pcDNA1-BombR and pcDNA3-M1R have been described¹. For stable expression of the M1R in Rat-1 cells the receptor was subcloned into pLXSN. proHB-EGF and the endothelin receptor were amplified by PCR from a MCF-7 or Rat-1 cDNA library and subcloned into pcDNA3-VSV or pcDNA3, respectively.

Cells and transfections

Rat-1 cells and COS-7 cells were grown and, respectively, infected and transfected, as described^{1,2,12,18}. Rat-1HERC cells have been described elsewhere¹. HEK 293 cells were grown in DMEM containing 10% FCS and transfections were carried out using the CaPO₄ method. CRM197 (10 μ g ml⁻¹, Sigma) or batimastat (BB-94), (5 μ M, British Biotech) were added 20 min before the respective growth factor. Tyrphostin AG1478 (250 nM, Calbiochem) and AG1295 (1 μ M, Calbiochem) were added 15 min before stimulation.

Immunoprecipitation and western blotting

The antibodies against human EGFR (108.1), SHP-2, SHC and Gab1 have all been characterized^{1,2,12,18}. Western blotting against the EP-R chimera was carried out using a rabbit polyclonal α -hPDGFR β antibody (Upstate Biotechnology). Cells were lysed and proteins immunoprecipitated as described¹. To precipitate the VSV-tagged HB-EGF, a monoclonal VSV antibody (P5D4, Boehringer) in combination with Protein G-Sepharose was used; HB-EGF was detected with antibody C-18 (Santa Cruz). Because of the small size of pro-HB-EGF and the processed form of HB-EGF, we used the tricine SDS-PAGE system as described²⁹.

Flow cytometry analysis

COS-7 cells were seeded in 6-cm dishes for 20 h, before being washed and cultured for a further 24 h in serum-free medium until being treated with growth factors as indicated. After collection, cells were incubated with goat α HB-EGF antibody (R&D systems) for

30 min on ice. After washing with PBS, cells were incubated with FITC-conjugated rabbit anti-goat antibody (Sigma) for 20 min on ice. Cells were analysed with FACScalibur (Becton Dickinson).

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p53 inhibition by the LANA protein of KSHV protects against cell death

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Kaposi's sarcoma-associated herpesvirus (KSHV), or human herpesvirus 8, has been implicated in the development of Kaposi's sarcoma (KS) and several B-cell lymphoproliferative diseases^{1–3}. Most cells in lesions derived from these malignancies are latently infected, and different viral gene products have been identified in association with lytic or latent infection by KSHV^{4,5}. The latency-associated nuclear antigen (LANA), encoded by open reading frame 73 of the KSHV genome, is a highly immunogenic protein that is expressed predominantly during viral latency, in most KS spindle cells and in cell lines established from body-cavity-based lymphomas^{6,7}. Antibodies to LANA can be detected in a high percentage of HIV-infected individuals who subsequently develop KS^{8,9}, although its role in disease pathogenesis is not completely understood. p53 is a potent transcriptional regulator of cell growth whose induction leads either to cell-cycle arrest or apoptosis. Loss of p53 function correlates with cell transformation and oncogenesis^{10,11}, and several viral oncoproteins interact with p53

and modulate its biological activity^{12,13}. Here we show that LANA interacts with the tumour suppressor protein p53 and represses its transcriptional activity. This viral gene product further inhibits the ability of p53 to induce cell death. We propose that LANA contributes to viral persistence and oncogenesis in KS through its ability to promote cell survival by altering p53 function.

We sought to determine whether LANA and p53 interacted because KSHV is associated with the transformation of several cell types. The complementary DNA encoding open reading frame 73 (orf73)/LANA from the KSHV-positive body-cavity-based lymphoma (BCBL)-1 cell line⁷ was subcloned into the mammalian expression vector pcDNA3.1. A truncated form of orf73/LANA encoding the first 440 amino acids (orf73Δ440) was generated to remove putative transcriptional regulatory domains, consisting of acidic and proline/glutamine-rich regions of the protein (Fig. 1a). *In vitro* translation followed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) analysis of orf73/LANA revealed a doublet band of 222–234K (relative molecular mass, M_r) (Fig. 1b, lane 1), as previously reported^{6,7}. Translation of the truncated mutant gave rise to a doublet of about 54–60K (Fig. 1b, lane 2). Immunofluorescence analysis of orf73Δ440, expressed as a fusion protein with a green fluorescent protein (GFP) at the amino terminus in mammalian cells, showed identical subnuclear localization to orf73/LANA (Fig. 1c).

We determined whether LANA could modulate p53 transcriptional activation using co-transfection experiments. Co-transfection of a p53 expression vector plasmid and a chloramphenicol acetyltransferase (CAT) reporter plasmid with consensus p53-binding sites in p53-null SAOS-2 osteosarcoma cells enhanced CAT

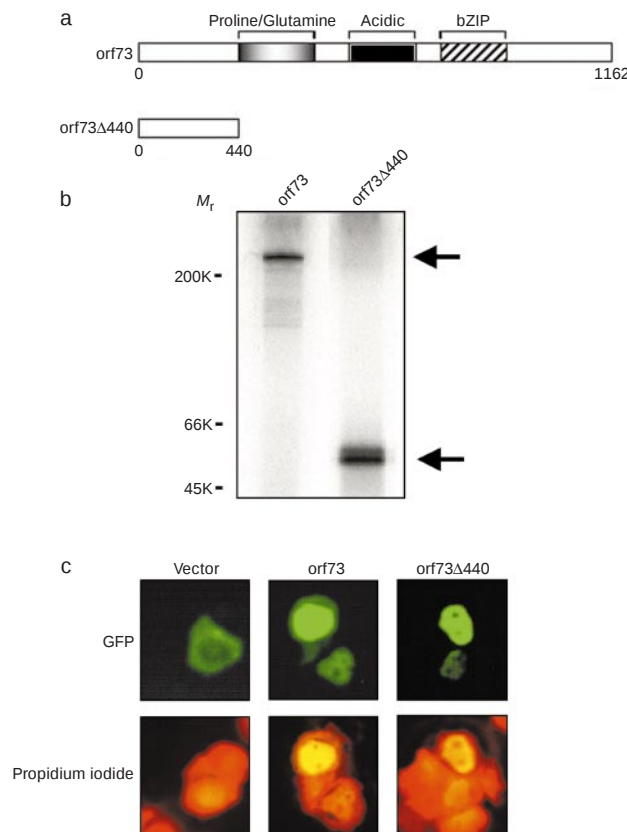


Figure 1 Orf73 of the KSHV genome encodes the 222–234K LANA. **a**, Representation of orf73/LANA derived from the BCBL-1 cell line. A truncated mutant of LANA was generated to remove the putative domains of the protein (orf73Δ440). **b**, *In vitro* translation of the expression vectors pcDNA 3.1 orf73/LANA and pcDNA3.1 orf73Δ440 proteins. Lane 1, a 222–234K orf73/LANA doublet band (upper arrow). Lane 2, a 54–60K orf73Δ440 doublet band (lower arrow). M_r , relative molecular mass. **c**, Transfection of 293 cells with

the expression vector pEGFP-C2 (vector) or with either pEGFP-orf73/LANA (orf73) or pEGFP-orf73Δ440 (orf73Δ440). Slide preparations (upper panels) were prepared and analysed by fluorescence microscopy using a Zeiss microscope with FITC filter sets. One set was counterstained with 1.0 $\mu\text{g ml}^{-1}$ propidium iodide and analysed under identical conditions (lower panels). Original magnification, $\times 100$.

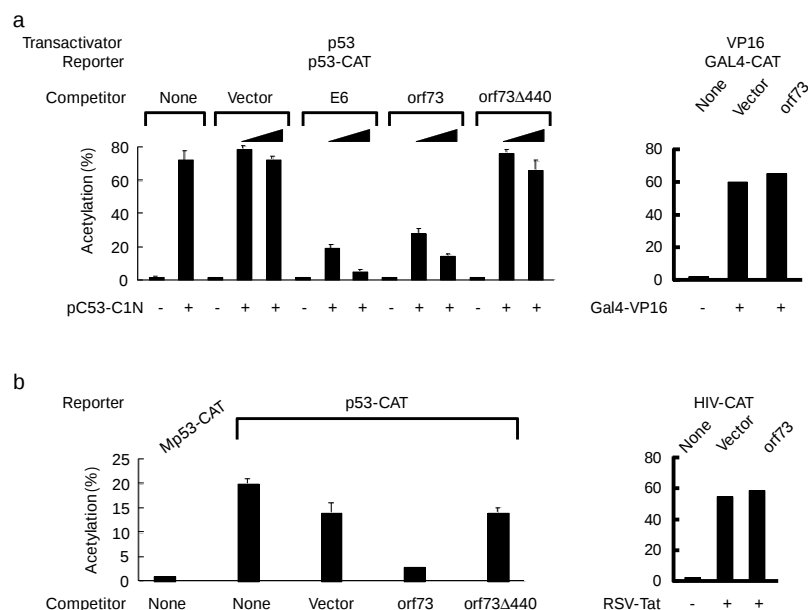


Figure 2 Inhibition of p53 transcriptional activity by orf73/LANA. **a**, The p53-null SAOS-2 cells were co-transfected with the reporter plasmid pG13-CAT (2 μ g) in the presence (+) or absence (–) of the expression plasmid pC53-C1N (2 μ g) together with 2–4 μ g of pcDNA3.1–E6, pcDNA3.1–orf73/LANA, pcDNA3.1–orf73Δ440 or the empty expression vector (left panel). See Methods for CAT assays. SAOS-2 cells were co-transfected with the GAL4 reporter plasmid pG5E4T–CAT (2.5 μ g) in the presence (+) or absence (–) of a GAL4–VP16 expression plasmid (2.5 μ g), together with 5 μ g of pcDNA 3.1–orf73/LANA

or the empty expression vector (right panel). **b**, Jurkat T leukaemia cells were transfected with 5 μ g of the reporter plasmid pG13-CAT or the negative reporter plasmid Mp53-CAT (left panel), or HIV-CAT in combination with RSV-Tat (0.5 ng) (right panel) together with 5 μ g of pcDNA3.1–orf73/LANA, pcDNA3.1–orf73Δ440 or the empty expression vector as indicated. Data are representative of three independent experiments, and error bars represent standard error values.

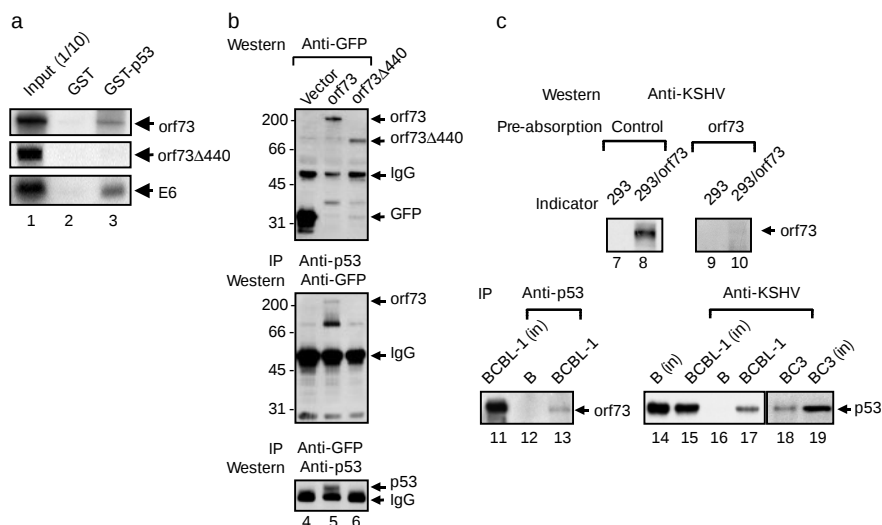


Figure 3 *In vitro* and *in vivo* interaction of transfected or endogenous orf73/LANA and p53. **a**, *In vitro* interaction of orf73/LANA with p53 protein evaluated in GST pull-down assays. The ability of *in vitro* translated orf73/LANA (top), orf73Δ440 (middle) or HPV-16 E6 (bottom) to be retained by GST (lane 2) or GST–p53 fusion protein (lane 3) was analysed by SDS–PAGE. **b**, *In vivo* interaction of the orf73/LANA with p53 was assessed by co-immunoprecipitation in SAOS-2 cells. Cells were co-transfected with the pC53-C1N expression plasmids (3 μ g) and the GFP expression plasmids (12 μ g) as indicated. Equal amounts of whole-cell extracts were immunoprecipitated with the p53 DO-1 antibody (middle) or an antibody directed against GFP (bottom) and analysed by western blot following SDS–PAGE. Western blot analysis of cell extracts (10%) was performed in parallel. The position of GFP fusion proteins detected by the GFP antibody is shown (top). **c**, Serum from KSHV-seropositive patient contains antibodies to orf73/LANA (upper panel). Nuclear extracts (20 μ g) from 293 cells transfected with the empty expression vector (lanes 7, 9) or pcDNA3.1–orf73/LANA (lanes 8, 10) were analysed by western blot

using an antiserum from a KSHV-seropositive patient. The KSHV serum pre-absorbed 293 cells transfected with vector (lanes 7, 8) or with orf73/LANA (lanes 9, 10). p53 immunoprecipitates from BCBL-1 cells contain LANA (lower left). Nuclear extracts (100 μ g) from primary B cells or BCBL-1 cells were incubated with the p53 DO-1 antibody (lanes 12 and 13, respectively), followed by western blotting with KSHV-positive antiserum. Analysis of BCBL-1 nuclear extracts (20 μ g) was carried out in parallel to show the position of orf73/LANA detected by the KSHV antiserum (lane 11). In reciprocal experiments, LANA immunoprecipitates from BCBL-1 contain p53 (lower right). Nuclear extracts (100 μ g) from primary B cells or BCBL-1 cells were incubated with a polyclonal antibody against LANA (lanes 16 and 17, respectively), followed by western blotting with the p53 DO-1 antibody. Analysis of B cells and BCBL-1 nuclear extracts (20 μ g) was carried out in parallel to show the position of p53 (lanes 14 and 15, respectively). Similar analyses were performed with a whole-cell extract (500 μ g) of BC3 cells (lane 18) and compared with input (50 μ g) of BC3 cell extract (lane 19), in, input.

expression ~80-fold (Fig. 2a, left panel). When the orf73/LANA was co-transfected with p53 and this reporter, however, p53 transcriptional activity was substantially inhibited; in contrast, orf73 Δ 440 had no effect on p53 function. Inclusion of a plasmid encoding the E6 oncoprotein of human papillomavirus (HPV) type 16, which is known to bind and promote the ubiquitin-dependent degradation of p53^{14,15}, was used as a positive control in these experiments and markedly diminished the ability of p53 to enhance CAT expression from the reporter plasmid (Fig. 2a, left). In addition, the expression vector without insert did not affect p53 transcription, excluding the possibility that this inhibition resulted from promoter competition. Moreover, LANA did not influence the transcriptional activity of an unrelated transactivator, a herpesvirus simplex VP16–GAL4 fusion protein, using a GAL4–CAT reporter plasmid in SAOS-2 cells (Fig. 2a, right). Transfection experiments were also performed in the Jurkat T-leukaemia line. When the p53 reporter plasmid was transfected into Jurkat cells, a 20-fold increase in CAT activity was observed, as compared with the negative control reporter plasmid, Mp53-CAT, which has mutated p53-binding sites (Fig. 2b, left). Expression of LANA markedly inhibited the transcriptional activity of endogenous p53, but had no effect on the capacity of an unrelated protein—human immunodeficiency virus Tat—to stimulate HIV-CAT activity in Jurkat cells (Fig. 2b, right panel). In addition, LANA did not influence the ability of cellular RelA(p65) to stimulate HIV-CAT activity (data not shown), further indicating its specificity for p53.

We then assessed whether the interaction between p53 and LANA is the molecular basis of p53 repression. p53 was expressed as a glutathione *S*-transferase (GST) fusion protein in bacteria, purified and precipitated with glutathione-agarose beads. *In vitro* translated orf73/LANA was retained by the GST–p53 fusion protein as compared with a GST control (Fig. 3a, upper panel, 25-fold increase relative to input compared with a GST negative control by quantitative imaging analysis). In contrast, no detectable binding was observed with the truncated orf73 Δ 440 (Fig. 3a, middle panel). The E6 protein from HPV-16 again served as a positive control (Fig. 3a, lower panel). The ability of LANA to interact with p53 *in vivo* was shown in two ways. The GFP–orf73/LANA fusion protein and p53 expression plasmids were co-transfected into SAOS-2 cells and co-immunoprecipitation analyses were performed. Incubation of cell extracts with a p53-specific antibody, DO-1, resulted in the co-immunoprecipitation of GFP–orf73/LANA but not truncated GFP–orf73 Δ 440 (negative control) (Fig. 3b, middle panel). Conversely, incubation with an antibody directed against GFP revealed co-immunoprecipitation of p53 with GFP–orf73/LANA but not GFP or GFP–orf73 Δ 440 (Fig. 3b, lower panel).

To confirm this interaction in KSHV-infected cells, antiserum from an HIV-seropositive patient with KS was used in western blot analysis on the KSHV positive BCBL-1 cell line. This serum contained high titres of antibodies specific for LANA, as judged by its ability to detect the characteristic doublet in orf73/LANA-transfected, but not control-transfected, 293 cells (Fig. 3c, lanes 7,

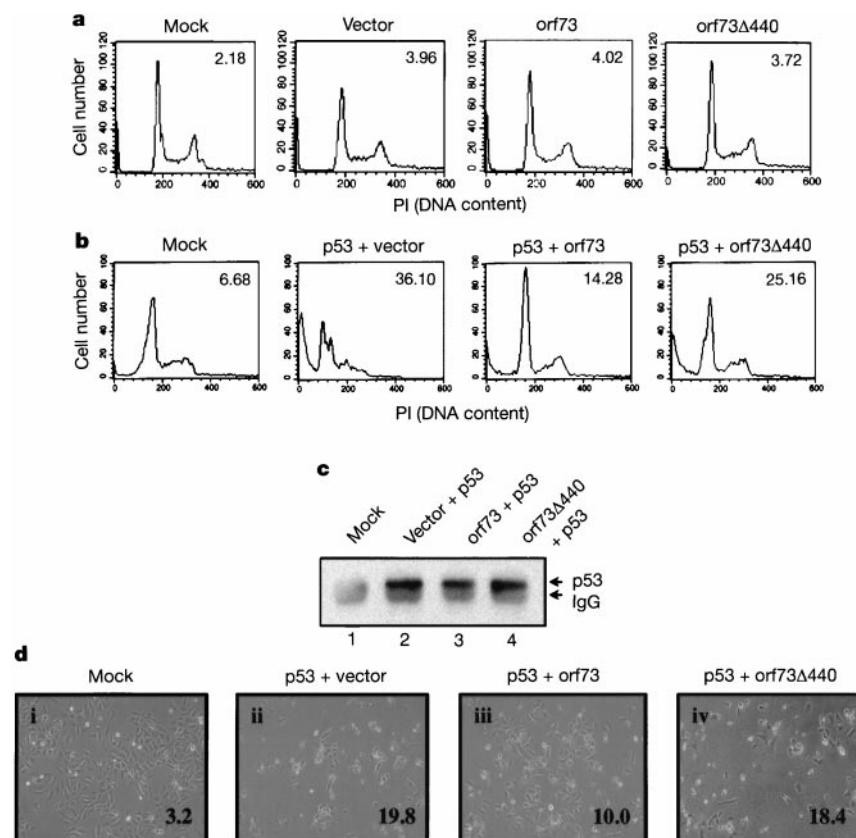


Figure 4 Repression of p53-mediated cell death by orf73/LANA. SAOS-2 cells were mock-transfected or transfected with 15 μ g of the empty expression vector, pcDNA3.1–orf73/LANA or pcDNA3.1–orf73 Δ 440 in the absence (**a**) or presence (**b**) of 5 μ g of the pC53-C1N expression plasmid. Adherent and floating cells were harvested and fixed with methanol, and cell-cycle progression was analysed by flow cytometry. DNA content was quantified by staining the cells with propidium iodide (PI). Numbers indicate the percentage of cells in sub-G1 phase of the cell cycle at 48 h after transfection. No significant changes were observed at 24 h after transfection in the presence of p53, with

1.88, 3.0, 4.2 and 5.96% of cells in sub-G1 in each group, respectively (data not shown).

c, Expression of p53 in the different SAOS-2 cell cultures was monitored by western blot analysis using the p53 DO-1 antibody. **d**, Cell morphology of SAOS-2 mock-transfected or co-transfected with the pC53-C1N expression plasmid and the empty expression vector, pcDNA3.1–orf73/LANA, or pcDNA3.1–orf73 Δ 440 as indicated, was analysed by phase contrast microscopy 72 h after transfection. Numbers indicate the percentage of annexin-V positive cells in the culture determined by flow cytometric analysis.

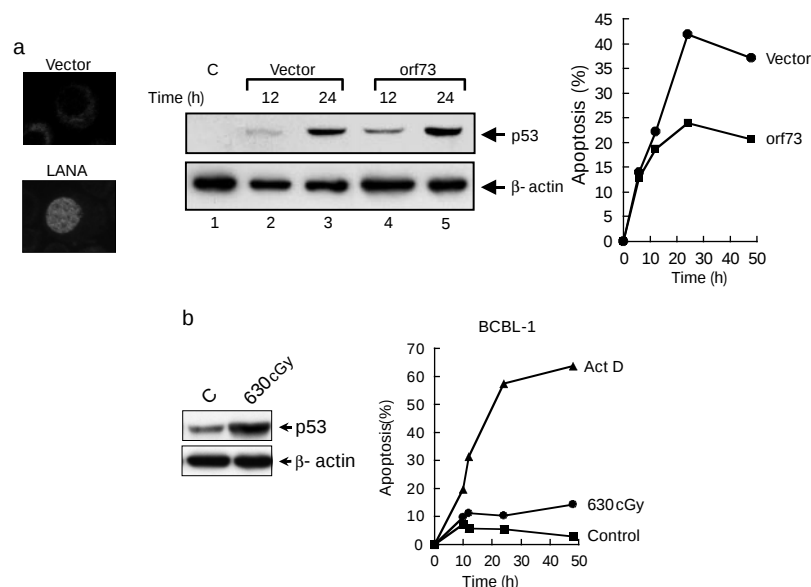


Figure 5 Inhibition of p53-mediated apoptosis in cell lines expressing LANA. **a**, Expression of LANA was monitored by immunofluorescence in NIH-3T3 cells stably transduced with a recombinant Moloney leukaemia retroviral vector lacking an insert or encoding orf73/LANA as described in Methods (left). NIH-3T3 stable transductants were starved (0% FBS), and western blot analysis was performed on equal quantities of whole-cell extract (50 μ g) using mouse p53 PAb 240 monoclonal antibody or β -actin antibody (arrows) to assess levels of induction of p53 following serum deprivation (middle). Apoptosis was assessed at various times using annexin-V staining assay (right). Control

(C) untransfected NIH-3T3 cells are indicated. **b**, BCBL-1 cells were exposed to 630 cGy ionizing radiation at room temperature, and western blot analysis was performed on equal quantities of whole-cell extract (50 μ g) using the p53 DO-1 or the β -actin antibody (arrows) 24 h after incubation (left). Control (C) untransfected NIH-3T3 cells are shown. Apoptosis was assessed at various times after incubation using annexin-V staining assay (right). Control BCBL-1 cells were incubated in the presence (triangles) or absence (squares) of actinomycin D (Act D, 10 μ M), or exposed to 630 cGy (circles).

8). Pre-absorption of the serum with orf73/LANA-transfected 293 cell lysates resulted in loss of reactivity (Fig. 3c, lanes 8 and 10). Immunoprecipitation of p53 followed by western blotting with this KSHV serum revealed a complex between LANA and p53 in the latently infected KSHV-positive BCBL-1 cells that was absent from uninfected primary B cells (Fig. 3c, lanes 12, 13). To confirm the specificity of this interaction, a reciprocal immunoprecipitation was performed using a specific anti-peptide polyclonal antibody directed against LANA, whose specificity was documented by pre-absorption as above. p53 was detected in anti-LANA immunoprecipitates of cell extracts from two latently KSHV-infected cell lines, BCBL-1 and BC3 (Fig. 3c, lane 17 and 18, respectively), but was absent from uninfected primary B cells (Fig. 3c, lane 16). Thus, LANA interacts with p53 in persistently infected cells.

To determine the potential biological effects of LANA on p53 function *in vivo*, its regulation of p53-induced cell death was examined. Overexpression of p53 in the SAOS-2 cell line induces apoptosis^{16,17}. LANA did not exert cytotoxic effects on transfected cells, as shown by analysis of cell-cycle progression and viability in the absence of p53 (Fig. 4a). Transfected SAOS-2 cells expressing p53 exhibited altered DNA content (by propidium iodide staining) indicative of increased apoptosis (Fig. 4b). Co-transfection of orf73/LANA significantly reduced the percentage of apoptotic cells (Fig. 4b, vector versus orf73 ($P < 0.04$) compared with orf73 Δ 440 ($P = 0.16$)), despite the fact that p53 levels were comparable (Fig. 4c). These observations were confirmed quantitatively by flow cytometric analysis for annexin-V staining assay (Fig. 4d). A significant increase in dying cells was evident in p53-transfected cultures compared to the control-transfected cultures (Fig. 4d). Co-transfection of orf73/LANA, but not its truncated form, orf73 Δ 440, provided substantial protection against this effect (Fig. 4d). To confirm the effect of LANA on endogenous wild-type p53, NIH-3T3 cells were transduced with recombinant Moloney leukaemia retroviral vectors encoding orf73/LANA, or a negative control vector with no insert, which also contained the neomycin-resistance

gene. Stable transductants were selected with G418 for 14 days, and expression of LANA was confirmed by immunofluorescence (Fig. 5a, left). Upon serum deprivation, a comparable increase in p53 levels was induced in both cell populations (Fig. 5a, middle), but apoptotic cell death was substantially reduced in LANA-transduced cells relative to the control vector-transduced cells (Fig. 5a, right). To examine the response to p53-mediated apoptosis in a cell line latently infected with KSHV, BCBL-1 cells, which constitutively express LANA and contain wild-type p53 as determined by DNA sequencing (data not shown), were subjected to γ -irradiation treatment. This treatment induced p53 expression (Fig. 5b, left panel), but no substantial increase in apoptosis was seen, in contrast to treatment with actinomycin D (Fig. 5b, right), which induces apoptosis independent of p53 activation¹⁸. Together, these data show that LANA inhibits p53 transcriptional function (Fig. 2) and its ability to induce apoptotic cell death *in vivo*.

As a consequence of their interactions with tumour suppressor proteins upon infection, viral oncoproteins can potentially disrupt cellular growth control to favour viral replication and cell transformation. Early analysis of KSHV genomic sequences revealed intriguing homologies to several oncogenic herpesviruses such as Epstein-Barr virus and herpesvirus saimiri^{1,19}. Of these proteins, a nuclear cyclin-D homologue (orf72/v-cyclin) inactivates a tumour suppressor protein, the retinoblastoma protein (Rb)²⁰. Interestingly, the orf72/v-cyclin and orf73/LANA of KSHV genomes are constitutively transcribed under standard growth conditions in BCBL cell lines and, thus, were identified as class I latent viral transcripts⁵ that may contribute to malignant transformation. Although antibodies to LANA are present in the infected individuals^{8,9}, its function has not been fully understood. Our findings suggest that LANA interacts with p53 and represses its transcriptional activity (Fig. 2). LANA does not influence the ability of p53 to bind DNA, nor does it induce degradation of p53 in a similar manner to HPV-16 E6 oncoproteins (data not shown). The orf73/LANA amino-acid sequence has motifs that can potentially modulate transcription

(Fig. 1a). Possibly it may also act as a transcription factor under different circumstances; however, the repression of p53 transcriptional activity is highly specific and is not due to squelching, and it also affects the ability of p53 to induce cell death *in vivo*. The interaction of LANA with p53 may be stabilized by additional factors, and LANA may act more generally as a transcriptional co-integrator that serves nuclear functions, including the maintenance of KSHV episomal DNA in persistently infected cells²¹. Nonetheless, the inhibition of p53-mediated apoptosis indicates that LANA may prolong cell survival and sustain KSHV viral persistence. p53 levels are increased in advanced cutaneous stages of AIDS-associated KS^{22,23}. The effect of LANA on p53, in combination with the effects of other putative viral oncogenes, is therefore likely to play a significant role in persistent viral infection and malignant transformation associated with KS. □

Methods

Plasmids

The reporter plasmid pG13-CAT contains 13 copies of the consensus p53-binding site upstream of the polyomavirus early promoter linked to the chloramphenicol acetyl-transferase (CAT) gene and has been described²⁴. The expression plasmid pC53-C1N, under the control of the cytomegalovirus immediate early promoter, contains the wild-type human p53 cDNA which includes a proline at polymorphic residue 72. Both the HIV Tat expression plasmid under the control of the Rous sarcoma promoter and the HIV-CAT reporter plasmid have been described^{25,26}. The expression plasmid GAL4-VP16 containing the activation domain of the HSV VP16 fused to the DNA-binding domain of GAL4 and the reporter plasmid pG₅E4T-CAT bearing five GAL4-binding sites upstream of an adenovirus E4 TATA-box have been described²⁷.

KSHV orf73/LANA and HPV-16 E6 cDNAs obtained, respectively, from the body-cavity-based lymphoma cell line, BCBL-1, and the cervical epidermoid cell line, CaSki, were amplified by PCR using the following primers: orf73/LANA; 5'-CTTTTATGT-CATTTC CTGTGG-3' (sense); 5'-CCAGATTCCCGAGGATGGCG-3' (antisense); E6; 5'-CTAGTCTAGACGTACAGCTGGGTTTCTCTACGTGTTTC-3' (sense); 5'-GATCG-GATC CACCATGTTTCAGGACCCACAGGAGCG-3' (antisense). PCR conditions used for amplification of DNA sequences were as described¹. The truncated mutant KSHV orf73Δ440 was generated by site-directed mutagenesis according to the manufacturer's protocols (Stratagene). Mutagenic primers used to introduce a stop codon at residue 440 of the orf73 coding sequence were as follows: 5'-TGGCTCCTGCTGCTGTGTTAACTTTGATGCTCAACGTTT-3' (sense); 5'-ACCGAGGACGACGACAACAATTGAAACCTACGAGTTGCAAAA-3' (antisense).

For *in vitro* expression, amplified PCR fragments were purified and ligated into the expression vector pCDNA3.1 according to the manufacturer's protocols (Invitrogen). For *in vivo* expression and immunobiochemical experiments, cDNAs were subcloned as an *HindIII*-*Apal* fragment into the expression vector pEGFP-C2 (Clontech) or as a *BamHI*-*XhoI* fragment into the expression vector pPGS-CITE-Neo (ref. 28).

In vitro transcription and GST pull-down assays

Coupled *in vitro* transcription-translation reactions were carried out using the TNT T7 system (Promega) in [³⁵S]methionine on 1 μg of plasmid DNA. Equal amounts of *in vitro* translated products were boiled in SDS gel loading buffer and resolved by SDS-PAGE with autoradiography.

For GST pull-down assays, GST or GST-p53 protein were absorbed to GST-coated beads, washed three times with sample buffer, incubated with *in vitro* translated products in a total volume of 100 μl at 4 °C for 2 h, washed and analysed as described²⁵.

Cell culture, stable transduction and CAT assays

The human embryonic kidney 293 cells, the p53-null SAOS-2 osteosarcoma cells and murine NIH-3T3 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), antibiotics and L-glutamine. Cell lines (293 and SAOS-2) were transfected using calcium phosphate²⁹. Jurkat T-leukaemia, BCBL-1 and BC3 (obtained from the American type culture collection) cells were maintained in RPMI 1640 medium supplemented with 10% FBS, antibiotics and L-glutamine. Jurkat cells were transfected using DEAE-dextran³⁰.

For stable transduction, recombinant Moloney leukaemia retroviral vectors were produced in 293 cells transfected with plasmids pNGVL4070A ENV, pNGVL-MLV gag-pol and pPGS-CITE-Neo orf73/LANA or pPGS-CITE-Neo vector as described²⁸. NIH-3T3 cells were immediately transduced with clarified supernatants, and stable transductants were isolated in the presence of G418 (GIBCO, BRL). The entire G418-resistant cell populations were maintained under selection for 14 days before analyses.

For CAT assays, SAOS-2 cells seeded at a density of 2 × 10⁶ cells were harvested 24 h after transfection, and lysates were prepared by three cycles of freeze thawing. Transfection efficiency was evaluated by transfecting parallel cultures with a GFP fusion expression plasmid. By immunofluorescence, 40% of cells were typically positive for GFP and p53 staining in these experiments. Jurkat cells (10 × 10⁶) were harvested 48 h after transfection. Protein concentrations were determined by the BioRad protein assay, and CAT assays were performed on equal quantities of protein extract as described³⁰. The acetylation of chloramphenicol was quantified by Phosphorimager.

Immunobiochemical analyses

For microscopic analyses of 293 cells transfected with pEGFP expression vectors, cell suspensions were washed twice with PBS 24 h after transfection, cytospun onto slides, and fixed in 3.7% paraformaldehyde/PBS for 30 min at room temperature. For microscopic analyses of NIH-3T3 stable transductants, cells were cytospun onto slides and fixed with cold methanol for 20 min at -20 °C. Slides were stained at 37 °C with a rabbit polyclonal antibody against LANA, washed twice and incubated with a fluorescein-conjugated goat anti-rabbit IgG antibody (ICN/Cappel). Analyses were performed using a Zeiss microscope with FITC and TRITC filter sets.

Western blot analyses

For immunoblot analyses, protein concentrations were determined by the BioRad protein assay, and equal amounts of nuclear extracts or whole cell lysates were incubated as indicated with monoclonal antibodies directed against p53 (antibody DO-1, Santa Cruz Biotechnology; antibody Pab 240, PharMingen), a monoclonal antibody directed against GFP (Clontech), a monoclonal antibody directed against β-actin (Sigma), or the polyclonal antibody directed against LANA. Immunoprecipitates were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blot analyses were performed using the indicated antibodies at a dilution of 1:500. Blots were incubated with the secondary antibodies and viewed by enhanced chemoluminescence performed according to the manufacturer (Amersham Life Sciences).

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Structure of a transiently phosphorylated switch in bacterial signal transduction

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Receiver domains are the dominant molecular switches in bacterial signalling^{1,2}. Although several structures of non-phosphorylated receiver domains have been reported^{3–8}, a detailed structural understanding of the activation arising from phosphorylation has been impeded by the very short half-lives of the aspartyl-phosphate linkages. Here we present the first structure of a receiver domain in its active state, the phosphorylated receiver domain of the bacterial enhancer-binding protein NtrC (nitrogen regulatory protein C). Nuclear magnetic resonance spectra were

taken during steady-state autophosphorylation/dephosphorylation, and three-dimensional spectra from multiple samples were combined. Phosphorylation induces a large conformational change involving a displacement of β -strands 4 and 5 and α -helices 3 and 4 away from the active site, a register shift and an axial rotation in helix 4. This creates an exposed hydrophobic surface that is likely to transmit the signal to the transcriptional activation domain.

'Two-component' regulatory systems, in which a phosphate is transferred from histidine in a sensor kinase to aspartate in the 'receiver domain' of a response regulator, dominate signal transduction in bacteria and also occur in archaea and eukarya^{1,2}. Forty-nine receiver domains can be identified in *Escherichia coli* by sequence homology. Two-component systems also control the expression of virulence and drug resistance factors in several pathogenic organisms⁹. A detailed structural understanding of the two-component signal cascade might help to develop new approaches for treating the increasingly multiple-antibiotic-resistant infections.

The response-regulator NtrC activates transcription by the σ^{54} -holoenzyme form of RNA polymerase¹⁰. Its phosphorylated amino-terminal receiver domain (P-NtrC^r) acts positively on the central output domain to allow oligomerization of the otherwise dimeric protein. Formation of NtrC oligomers is required for ATP hydrolysis, which leads to transcriptional activation. We have previously determined the structure of the unphosphorylated receiver domain of NtrC (residues 1–124) (NtrC^r) (ref. 5) and mapped chemical shift differences in constitutive mutant forms, which have partial activity without being phosphorylated¹¹. NMR studies of phosphorylated chemotaxis protein Y (CheY) have been reported^{12,13} but the low stability of the phosphoaspartate linkage precluded a full structural analysis.

To determine a protein structure using NMR, samples must be stable for periods of days. Because the lifetime of the phosphoaspartate in P-NtrC^r is only a few minutes at 25 °C, two modifications to the usual approaches were required for study of P-NtrC^r: a high steady-state level of phosphorylation was maintained *in situ* with a low-molecular-weight phosphodonor; and multiple three-dimensional NMR spectra were collected, each on a newly prepared sample, and then added to enhance the signal to noise ratio. These methods use the intrinsic property of receiver domains to catalyse their own phosphorylation from low-molecular-weight phosphodonor¹⁴. A large excess of carbamoylphosphate was used as phosphodonor and three-dimensional NMR data sets were collected during turnover. Phosphorylation was initiated by adding Mg²⁺. The protein could be maintained more than 95% phosphorylated for 36 hours. The activity of P-NtrC^r under these

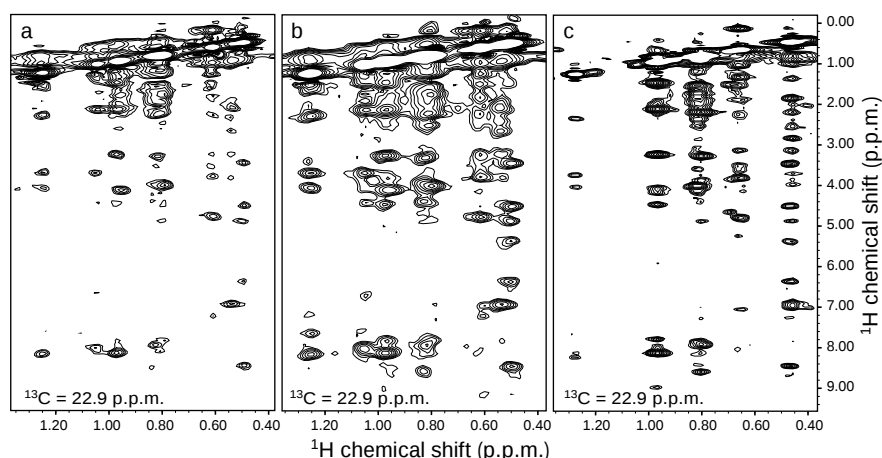


Figure 1 Gain in signal-to-noise ratio by adding multiple three-dimensional NMR data taken on different samples. **a–c**, Two-dimensional planes of ¹³C-edited three-dimensional NOESYs of a single three-dimensional spectrum of P-NtrC^r (**a**), a data set with

four added individual spectra of P-NtrC^r (**b**) and NtrC^r (**c**) are compared. The decreased resolution in the second dimension of **a** and **b** is due to the shorter experimental time for the phosphorylated form.

conditions was verified in assays of transcriptional activation; phosphorylation in a unique position was verified by mass spectrometry, which also confirmed the level of phosphorylation. The protein concentration used in the NMR experiments was limited to 0.3 mM because P-NtrC^r tended to aggregate at higher concentrations, as detected by a loss of signals in triple resonance experiments. To obtain a sufficient signal-to-noise ratio under those experimental conditions, we added multiple three-dimensional data sets (Fig. 1). Careful matching of the sample and spectrometer conditions yielded final data sets of a quality comparable to the spectra of NtrC^r. More than half of the signals that were clearly detectable after adding four data sets could not be detected in a single data set of a three-dimensional ¹³C-edited NOESY spectrum. For P-NtrC^r, assignments were obtained using triple resonance coherence transfer, and ¹⁵N- and ¹³C-resolved coherence transfer and NOE experiments. Distance restraints were generated from the NOE data.

The structure of P-NtrC^r is shown in Fig. 2, with structural statistics presented in Table 1. The overall topology of receiver domains—the β5/α5 fold—is preserved in the phosphorylated form. The active site lies in a cleft at the carboxy-terminal end of the parallel β-sheet, with D54, the site of phosphorylation, located at the end of strand 3. Although helix 4 is locally well defined, its position relative to the core of the protein is less well characterized because there are a smaller number of long-range NOEs between this helix and the core in P-NtrC^r as compared with NtrC^r. The relative disorder of this helix can be accounted for by the structural rearrangements triggered by phosphorylation (discussed below).

To enable a more precise analysis of the conformational switch upon phosphorylation, we further refined the initial structure⁵ of the unphosphorylated form. The main features of the phosphorylation-induced activation were assessed by comparing the structures in the inactive and active state after superposition of the backbone atoms of those residues that did not show significant chemical shift changes upon phosphorylation (Fig. 3). Significant structural alterations occur on one face of the molecule, including the region from α-helix 3 to β-strand 5. We previously identified this region, the '3445 face', as the potential 'switch region' by comparing wild-type NtrC^r with constitutively active mutant forms¹¹. Phosphorylation induces a displacement of β-strands 4 and 5 and α-helices 3 and 4 away from the active site. There is also a substantial

rearrangement of the loop above the active site, residues 55–64, as well as the loops leading into and out of helix 4. Interestingly, amide signals in these loops could not be observed in NtrC^r as a result of exchange broadening, but are readily detectable in P-NtrC^r. This suggests that these loops fluctuate in the inactive state and become more restricted in their conformational space upon phosphorylation. In addition to a substantial tilt, helix 4 shifts register by about two residues from the N to the C terminus of the helix, corresponding to more than half a turn. This change in the residues defining helix 4 is supported by changes in chemical shifts of the Cα carbons when NtrC^r is phosphorylated. The global displacement in combination with the register shift results in a rotation of about 100° about the helical axis. As a consequence, the side chains of L87, A90 and V91 rotate from the side facing the core of NtrC^r to the outside. The rotation of hydrophobic side chains probably explains the fact that deviations within the family of conformers in P-NtrC^r were larger in helix 4 than in other regions. Whereas helix 4 is well positioned in NtrC^r as identified by several NOEs between the methyl groups of L87, A90 and V91 and the core of the protein, these interactions do not occur in P-NtrC^r; instead, the above residues are poised to contact the output domain. New interactions, identified by NOEs between residues A89 (α4) and M81 (β4), and between A93 (α4) and I80 (β4) and Y101 (β5), position helix 4 with respect to the core of P-NtrC^r. The re-orientation of the hydrophobic side chains results in the formation of an exposed hydrophobic surface on helix 4 (Fig. 4a). This hydrophobic patch on P-NtrC^r might explain the tendency of the phosphorylated domain to aggregate non-specifically at higher protein concentrations, which was not observed with the unphosphorylated domain. We verified the monomeric state of P-NtrC^r by NMR diffusion experiments, ultracentrifugation and NMR relaxation studies (D.K. *et al.*, manuscript in preparation). The differences in the self-diffusion constants and the overall correlation times between P-NtrC^r and NtrC^r can be attributed to the measured difference in viscosity of the two samples. The above experiments also showed that aggregation of P-NtrC^r was eliminated at 0.3 mM protein concentration.

We propose that the phosphorylation-dependent hydrophobic surface of α-helix 4, which is solvent-exposed in isolated P-NtrC^r, serves to interact with the transcriptional activation domain in the intact protein. That such an interaction occurs is indicated by three lines of evidence. First, NtrC^r acts positively, that is, removing the

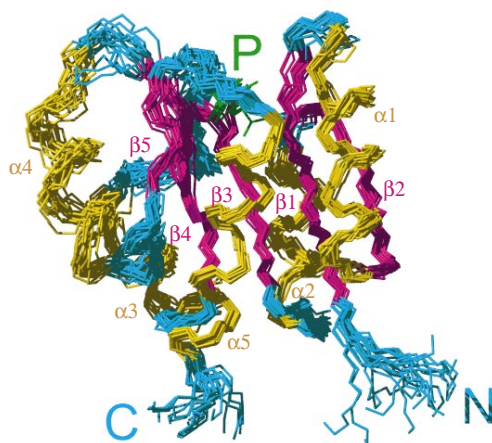


Figure 2 The structure of the phosphorylated receiver domain of NtrC. The 20 conformers with the lowest DYANA²⁸ target function were superimposed using the backbone atoms N, Cα and C' of residues 3–83 (β1–loop 3), 87–96 (α4) and 98–122 (β5–α5), the best-defined areas (global backbone displacement <2.0 Å). Helices are coloured in yellow, strands in magenta and loops and chain termini in cyan. The side chain of the phosphorylated D54 is shown in green. The secondary structure elements are labelled as they are referred to in the text.

Table 1 Parameters characterizing the NMR structure determination of P-NtrC^r and NtrC^r*

Parameter	P-NtrC ^r	NtrC ^r
Number of NOEs	3,095	3,044
Number of upper distance constraints		
Total	1,301	1,768
Intraresidue	374	355
Short-range	405	464
Medium-range	228	445
Long-range	294	504
DYANA target function (Å ²)†	5.6 ± 0.8	5.6 ± 1.1
Number of distance constraint violations >0.2 Å†	16 ± 4	18 ± 6
Largest distance constraint violation (Å)†	0.49 ± 0.11	0.54 ± 0.08
Sum of distance constraint violations (Å)†	20.6 ± 1.5	21.2 ± 2.2
r.m.s.d. to the mean for N, Cα and C' (Å)‡	0.7 ± 0.07	0.60 ± 0.09
r.m.s.d. to the mean for heavy atoms (Å)‡	1.23 ± 0.12	1.15 ± 0.10
r.m.s.d. to the mean for N, Cα and C' for secondary structure (Å)§	0.52 ± 0.08	0.53 ± 0.13
r.m.s.d. to the mean for N, Cα and C' of helix 4 (Å)¶	0.61 ± 0.13	0.42 ± 0.13

* The 20 conformers with the lowest DYANA target function were used starting from 40 randomized starting structures.

† The values given are average ± standard deviation over the 20 conformers.

‡ R.m.s.d. values are average ± standard deviation over the 20 conformers given for the best-defined residues—those with global displacement of the backbone atoms less than 2 Å: 3–83, 87–96, 98–122 for P-NtrC^r; and 3–54, 66–93 and 98–122 for NtrC^r.

§ Excluding helix 4.
¶ Residues 85–95 for P-NtrC^r and 83–93 for NtrC^r.

r.m.s.d., root mean square deviation.

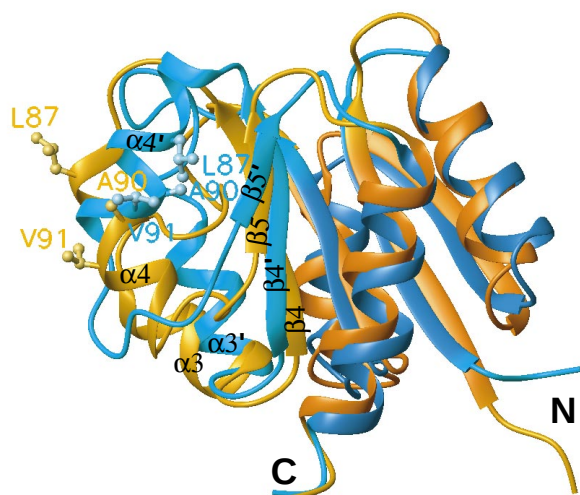


Figure 3 Molecular switch upon phosphorylation of D54 in NtrC'. Superimposed ribbon structures of P-NtrC' (yellow/orange) and NtrC' (cyan/blue), generated using the conformer closest to the mean structure for each. The molecule has been rotated by 20° about the vertical axis with respect to Fig. 2 to emphasize the regions of structural differences between the two forms. Structures were superimposed using residues 4–9, 14–53 and 108–121, which are indicated in darker colours (orange and blue). The regions of greatest difference are highlighted (yellow and cyan, the switch area) and the corresponding secondary structure elements are labelled, with the prime indicating the unphosphorylated form. Upon phosphorylation of D54, β -strands 4 and 5 and α -helices 3 and 4 tilt to the left—away from the active site. In addition, a register shift by about two amino-acid residues from the N to the C terminus and a rotation by about 100° about the helical axis are induced in helix 4 upon phosphorylation. The rotation results in a change in orientation of the hydrophobic side chains in helix 4 from the inside to the outside.

receiver domain does not substitute for phosphorylation. This implies that a new interaction between domains must be present after phosphorylation. Second, electron paramagnetic resonance studies on full-length NtrC indicate that there is a phosphorylation-induced interdomain interaction between helix 4 in the receiver domain and the remainder of the protein¹⁵. Third, cleavage by iron chelates tethered at positions 86 and 89 in helix 4 confirm the occurrence of a phosphorylation-dependent interdomain interaction (J. Lee *et al.*, manuscript in preparation).

Although the resolution in the active site of P-NtrC' is not sufficient to allow us to address the question of the mechanism of activation of receiver domains in detail, we can propose possible roles for active site residues based on the structural rearrangements we observed. In the first β -strand of receiver domains two aspartates are highly conserved and are usually followed by aspartic acid or glutamic acid (D10, D11 and D12 in NtrC) (ref. 16). In the three-dimensional structure, these are near the active site D54. By analogy to crystal structures of homologous CheY (refs 17, 18), the probable magnesium-binding site in NtrC' can be identified as the side chains of D10 and D54 and the backbone carbonyl of R56. The binding of phosphate to the active-site aspartate eliminates one of the magnesium ligands, phosphate occupying the space where the magnesium was. This would give rise to two effects. First, the change of the position of Mg^{2+} would lead to an electrostatic repulsion among the side chains of D10, D11 (and perhaps D12) and the phosphate group attached to D54, which may separate D54 from the other aspartates. Second, the backbone at R56 would be released, enabling the rearrangement of the loop connecting $\beta 3$ to $\alpha 3$ and in turn allowing reorganization of the '3445' face of the molecule. This idea

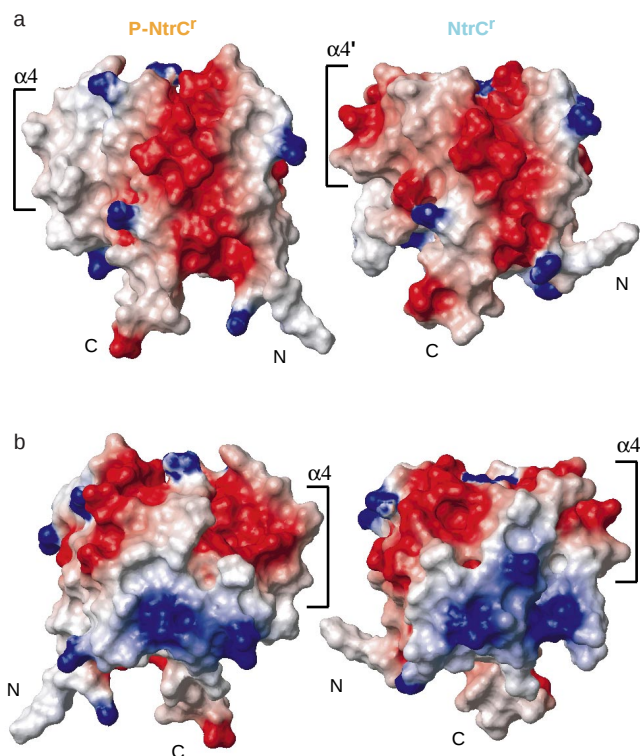


Figure 4 Formation of an exposed hydrophobic surface on helix 4 upon phosphorylation. Electrostatic surface potentials (calculated in MOLMOL²⁹) are shown for P-NtrC' (left) and NtrC' (right), with negative and positive potential values shown in red and blue, respectively. The molecules are oriented as in Fig. 3 (a) and rotated by 180° about the vertical axis (b). The hydrophobic face in helix 4 in the phosphorylated protein is shown in white (a). Molecular structure representations for Figs 2–4 were generated with the programme MOLMOL²⁹.

is supported by the fact that the mutational substitution D54E, which yields a low level of constitutive activity, moves the charge nearer to where it would be in P-NtrC' and results in loss of magnesium binding¹¹. A moderate flexibility for parts of the receiver domain structure has been suggested previously (for NtrC', Spo0F and CheY), particularly for structures studied in the absence of Mg^{2+} (refs 5, 19, 20). Owing to electrostatic repulsion and lack of backbone anchoring in the $\beta 3$ – $\alpha 3$ loop in the absence of magnesium, it seems likely that the structure fluctuates between inactive and active conformations, with the inactive predominating. With the D54E substitution, or mutational substitutions in helix 4 (D86N and/or A89T), the equilibrium is shifted to include a larger fraction of the active form¹¹, and upon phosphorylation is driven essentially completely to it. In NMR studies of NtrC' (D86N/A89T), the region around helix 4 is poorly defined, and showed a complete lack of long-range NOEs (D.K. *et al.*, manuscript in preparation), consistent with a fluctuating structure.

Extensive genetic studies have been done on many different receiver domains to detect regions involved in activation. The difficulties in interpreting these genetic studies in the light of the present structure are twofold. First, mutations can result in structural changes distant from their site of occurrence. Therefore the positions of mutational substitutions do not necessarily reveal the site(s) of structural rearrangement upon phosphorylation. Second, different receiver domains of the superfamily modulate the function of a variety of output domains/signalling pathways with a high degree of specificity, and it is not known whether phosphorylation of different receiver domains always induces similar structural changes. The molecular architecture of unphosphorylated receiver

domains is highly conserved. To generate the observed specificity among response-regulator–sensor-kinase pairs, distinct subsets of the structural changes may be exploited. Alternatively, phosphorylation could result in different structural changes.

The only published data to our knowledge on phosphorylation-dependent conformational changes in a receiver domain are those for CheY (refs 12, 13). Chemical shift changes of amide signals in CheY upon phosphorylation were seen in similar regions to those in P-NtrC^r (ref. 13), but in some different areas as well. Interpretation of such chemical shift mapping data with respect to structural rearrangements is complicated by the fact that there is no easy correlation between the size of chemical shift perturbations and structural changes. In unphosphorylated Spo0F, an inactive receiver domain, regions with slow (microsecond to millisecond) dynamics were inferred to be involved in conformational changes upon phosphorylation²⁰. Most of these are in loop regions close to the active site, and as noted above may reflect a structural fluctuation in the absence of Mg²⁺. In addition, slow motions were detected in α 1, where we did not observe structural alterations in P-NtrC^r. Further studies of phosphorylated Spo0F will be necessary to test the importance of those motions for function.

Our data provide the first detailed view of the conformational changes that occur upon phosphorylation (activation) of a receiver domain. Although protein phosphorylation is one of the most common mechanisms of post-translational regulation²¹, the structural basis for this type of regulation has been elucidated for relatively few proteins, and all of these are stably phosphorylated (on serine, threonine or tyrosine). The diversity of conformational changes triggered by phosphorylation ranges from small local changes²² to larger structural changes within a domain^{23,24} or subunit reorientation^{25,26}. Phosphorylation of *E. coli* isocitrate dehydrogenase does not alter the structure substantially, but inhibits substrate binding directly because of steric and electrostatic hindrance²². The primary event of phosphorylation in different kinases is a conformational change of an activation loop carrying the phosphorylation site(s)^{23,24}. The mechanisms that trigger phosphorylation-dependent activation of mammalian and yeast glycogen phosphorylases differ^{25,26}; however, the final event—a change in the subunit interface—is similar. That the activation mechanisms are distinct is not surprising because the phosphorylation sites are different. In receiver domains, the opposite might be true. Owing to the highly conserved phosphorylation site, the activation mechanisms may be similar among receiver domains but the final output may vary.

The challenge in elucidating the conformational switch in the receiver domain of NtrC was the transient stability of its phosphorylated form. We made use of its autophosphorylating activity and acquired data for structural analysis on the working enzyme. To our knowledge, this is the first solution structure of a protein in a short-lived state. The techniques used here should be suitable for the structural characterization of other transient species, thereby expanding the applicability of NMR spectroscopy. □

Methods

Sample preparation

Uniformly ¹⁵N and ¹⁵N/¹³C-labelled NtrC^r was prepared as described¹¹. The optimal initial sample conditions were 0.3 mM P-NtrC^r, 200 mM sodium phosphate pH 6.75, 50 mM MgCl₂, 200 mM carbamoylphosphate and 10% D₂O. Phosphorylation was initiated by adding MgCl₂. The level of phosphorylation was measured by ¹⁵N-¹H HSQC after each 36-hour experiment and found to be at least 95%. After complete consumption of carbamoylphosphate, a ¹⁵N-¹H HSQC spectrum identical to that of (unphosphorylated) NtrC^r was obtained. For the refinement of NtrC^r, new data were taken in 50 mM sodium phosphate, pH 6.75, 10% D₂O.

Oligomeric state

To rule out that the increased ionic strength in the sample of P-NtrC^r caused changes in chemical shift or structure, ¹⁵N-¹H HSQC and ¹³C-¹H HSQC spectra were taken for NtrC^r in 200 mM sodium phosphate pH 6.75, 200 mM carbamoylphosphate and 10% D₂O (with no magnesium to inhibit phosphorylation). No changes of the spectrum were

observed. The ratio of the viscosity between the buffers used for structure determination of P-NtrC^r and NtrC^r was 1.4, measured with an Ostwald viscometer. Self-diffusion constants were measured by pulsed-field gradient NMR as described²⁷, yielding values of 1.53 and 1.10 (10⁻⁶ cm² s⁻¹), respectively. In equilibrium sedimentation experiments using a Beckman XL-I ultracentrifuge, both P-NtrC^r and NtrC^r showed monomeric behaviour in a protein-concentration range between 15 and 300 μ M. The overall correlation times for P-NtrC^r (10.7 ns) and NtrC^r (6.5 ns) in the buffers described above were determined from the model-free analysis using ¹⁵N relaxation data. All data are consistent with P-NtrC^r being monomeric, and tumbling more slowly than NtrC^r because of increased solvent viscosity.

NMR spectroscopy and structure calculations

NMR spectra were acquired at 25 °C on either a Bruker DMX 600 or 750 NMR spectrometer (numbers in parentheses indicate the number of data sets which were co-added): ¹⁵N-¹H HSQC, ¹³C-¹H CT-HSQC (aliphatic), ¹³C-¹H CT-HSQC (aromatic), ¹H-¹H NOESY in D₂O, HNCO, HNCA (2), C(CO)NH (2), HCCH-TOCSY (4), ¹⁵N-edited TOCSY (4), ¹⁵N-edited NOESY (4) and ¹³C-edited NOESY (4). (NOESY, nuclear Overhauser enhancement spectroscopy; TOCSY, total correlation spectroscopy, CT-HSQC, constant time heteronuclear single quantum correlation spectroscopy). All NOESY mixing times were 100 ms. The experimental conditions were closely matched between the individual samples of the same three-dimensional experiment. The free induction decays of the data sets were added before Fourier transformation. Upper distance constraints were calculated from NOESY cross-peak intensities using the CALIBA²⁸ procedure. The structure calculations were performed using simulated annealing in torsion-angle space in DYANA²⁹. The phosphoryl group was modelled using a modified aspartate residue at position 54 with the phosphoryl group covalently attached. As no experimental constraints are available restraining the phosphoryl group, only van der Waals interactions involving the phosphoryl group were active during the structure calculation with the distance geometry programme DYANA. The programme MOLMOL²⁹ was used to analyse the structures.

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errata

A Chinese triconodont mammal and mosaic evolution of the mammalian skeleton

Ji Qiang, Luo Zhexi & Ji Shu-an

Nature **398**, 326–330 (1999)

The names of these authors are listed as the Chinese would write them and not according to *Nature's* convention, that is with the family name first and the given name last. Correct citations of this paper should therefore refer to the authors as Ji, Q., Luo, Z.-X. and Ji, S.-A. □

Small-bandgap endohedral metallofullerenes in high yield and purity

S. Stevenson, G. Rice, T. Glass, K. Harich, F. Cromer, M. R. Jordan, J. Craft, E. Hadju, R. Bible, M. M. Olmstead, K. Maitra, A. J. Fisher, A. L. Balch & H. C. Dorn

Nature **401**, 55–57 (1999)

In the second paragraph on page 55, the third sentence is misleading and should read “The yields even exceed those of the empty-cage C₈₄” (without the erroneously inserted text “...the usually most abundant species...”). □

Acknowledgements

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Correspondence and requests for materials should be addressed to D.K. (e-mail: dking@brandeis.edu). Coordinates for the NMR structures of NtrC^f and P-NtrC^f have been deposited in the Research Collaboratory for Structural Bioinformatics Protein Data Bank under the accession codes 1DC7 (NtrC^f) and 1DC8 (P-NtrC^f).

Saccharomyces cerevisiae telomerase is an Sm small nuclear ribonucleoprotein particle

Anita G. Seto, Arthur J. Zaugg, Suzanne G. Sobel, Sandra L. Wolin & Thomas R. Cech

Nature **401**, 177–180 (1999)

The gel shown in Fig. 3a of this Letter should have had lanes 2–10 labelled I, S, B (in that order, three times, from left to right) for “input”, “supernatant” and “bound”, respectively. □

Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14

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Units for the x-axis of Fig. 5b–g should be pmol kg^{−1} (and not nmol kg^{−1} as published). In Fig. 5f, the right numeral should be 1,000. □